Bacillus systems exporting folded proteins and folding exported proteins

Goosens, Vivianne Jacoba

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2013

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Stellingen

1. Looking at the same data from different angles can give useful and different results (Illustrated in the hypothesis driving or driven approaches in Chapter 2 of this thesis)

2. Just because you don't see it, doesn't mean its not there (Illustrated by the flaws of Mass Spectrometry - this thesis).

3. TatAc assists TatAy with regard to EfeB translocation in *Bacillus subtilis* (Chapter 3 of this thesis)

4. TatAc of *B. subtilis* could represent an evolutionary intermediate of *Escherichia coli's* TatA-TatB proteins (Chapter 3 of this thesis)

5. Rieske proteins are Tat-dependent cargo proteins in a number of prokaryotes, including *B. subtilis* (Chapter 2 of this thesis)

6. The successful translocation of Rieske proteins is dependent on both the correct disulphide bond assisted-folding and co-factor insertion, but only disulphide bond mutations result in rapid Rieske protein degradation (Chapter 3 of this thesis).

7. Residues in the amphipathic helix region of *B. subtilis* TatAy interact specifically with different cargo proteins (Chapter 3 and 4 this thesis)

8. Tat-associated phenotypes caused by environmental salinity are cargo protein-specific and not due to the Tat pathway directly (Chapter 4 this thesis).

9. *B. subtilis* cells exposed to environmental salt stress unveil a BdbCD-associated phenotype (Chapter 5 this Thesis)

10. The 'circle of live' is neatly illustrated by the relationship between QcrA and the Tat pathway. The cytochrome bc_1 complex, specifically QcrA, is dependent on Tat for maturation, while Tat is dependent on the proton motif force generated by the cytochrome bc_1 complex (this thesis).

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Ruben A.T. Mars

"...He misses the feeling of creating something out of something. That's right – something out of something. Because something out of nothing is when you make something up out of thin air, in which case it has no value. Anybody can do that. But something out of something means it was really there the whole time, inside you, and you discover it as part of something new, that's never happened before..."

From Etgar Keret's short story 'Suddenly a knock on the door'.

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Tabel of Content

Thanks to the many inspiring women in my life

'MFB only'

Chapter 1

Introduction and Scope of this Thesis

This Chapter has been adapted from:

Vivianne J. Goosens, Carmine G. Monteferrante, and Jan Maarten van Dijl (2013) The Tat system of Gram-positive bacteria, *Biochim. Biophys. Acta*, in press

Introduction

The transport of molecules across the cytoplasmic membrane is fundamental for the life of any cell, whether prokaryotic, archaeal or eukaryotic. Import of molecules from the outside to the inside of cells is essential for nutrition and provides cells with vital information concerning the ever-changing conditions in which they find themselves. Although for differing reasons, the export of molecules from the inside to the outside of cells is equally important. In the first place, this is needed to dispose of waste products. However, export processes are also indispensable for assembly of the transport machinery for nutrient intake, sensing of environmental changes and insults, cell-cell interactions and, in the case of pathogens, the targeting of virulence factors to a susceptible hosts. The latter export processes have in common that they involve the movement of proteins from the site of synthesis – usually the cytoplasm - to the membrane and other extracytoplasmic locations. Since proper folding is crucial for the activity and stability of proteins, the research described in this thesis has been focused on the export of folded proteins and the folding of exported proteins.

There are a number of pathways involved in protein export, of which the general secretion pathway (Sec) is universally conserved (1-4). Proteins that follow the Sec pathway are exported in an unfolded state. Hence, such proteins need to fold post-translocationally, and separate systems have evolved to specifically chaperone this folding process. Amongst the conserved catalysts of post-translational protein folding, the so-called thioldisulphide oxidoreductases (TDORs) take an important place, as they are required for the formation of disulphide bonds that give rigor to protein structure (5, 6). Intriguingly, a few protein export pathways can transport fully folded proteins. One such pathway that is present in bacteria and the thylakoids of plant chloroplasts is the so-called twin-arginine translocation (Tat) pathway (7-11).

Bacillus subtilis is a Gram-positive bacterium with the ability to secrete large amounts of protein directly into its growth medium. Many secreted enzymes of *Bacillus* species, such as *B. subtilis*, are enzymes of high commercial value. Accordingly, *B. subtilis* has become a workhorse for the bio-industrial production of proteins, a development that was greatly aided by the Generally Regarded as Safe (GRAS) status of this organism (12-14). At the same time, the high secretion capacity of *B. subtilis* raised interest with respect to fundamental mechanistic aspects, in particular the usage of different secretion pathways and the folding of secreted proteins. This was a primary incentive for the research described in this thesis which addressed a *B. subtilis* system involved in the export of folded proteins – the Tat pathway – and

a *B. subtilis* systems involved in the oxidative folding of secreted proteins – the Bdb system.

Introduction to the Tat System

The Twin-arginine translocation (Tat) pathway is found in the membrane of bacteria, archaea and in the thylakoids of chloroplasts. It is unique in that before proteins are moved from the cytoplasm into or over the membrane, they are correctly folded and, if appropriate, co-factors are attached (15-21). The cargo proteins are globular and the pathway must allow for variable-sized cargo and, therefore, a range of potential pore sizes (22). A second distinctive identifier of the Tat system is the eponymous motif containing twin-arginine residues in the signal peptide of proteins destined for transport via this pathway.

The Tat system is most studied in the Gram-negative bacterium *Escherichia coli*. However, in recent years, the understanding of the Tat system in Gram-positive bacteria, such as *B. subtilis* and corynebacteria has expanded extensively. Combined, these studies have highlighted a number of strong similarities and some interesting differences between the bacterial Tat systems. Components of the *B. subtilis* Tat system are able to functionally replace all components of the Tat system in *E. coli* (23, 24), they all have similar signal peptide requirements (25-27), and there are numerous examples of interspecies crossover with regard to substrates (24, 28-32). Differences seem to come in at the quality control and chaperone level. These similarities suggest that, at a fundamental level, the Tat system follows similar mechanisms in both Gram-positive and Gram-negative bacteria.

The Core Tat System

Cargo proteins are destined for the Tat system by virtue of specific N-terminal signal peptides. The signal peptide of the Tat system is similar to that of the Sec system, the main bacterial protein secretion pathway, but there are several distinctions between the two. Signal peptides are generally composed of three defined regions; a N-terminal domain, a hydrophobic region and a polar C-terminal domain containing the signal peptidase recognition site (14, 33). On average, the hydrophobic region of Tat signal peptides is larger and less hydrophobic than that of Sec signal peptides (34). The canonical N-terminal twin-arginine motif is S/T-RRxFLK (with x referring to a polar amino acid) and it is central to Tat-dependent translocation (11, 35).

There are a few instances where Tat-dependently translocated proteins do not have signal peptides. However, in these cases the cargo protein is associated with a second Tat signal peptide-containing protein and a piggyback or hitchhiker mechanism allows for its translocation (36, 37).

The current consensus model for the Tat system involves a docking complex and a pore complex. In essence this requires two types of proteins: a large integral membrane protein named TatC and a small membrane protein named TatA. Multiple TatA-like proteins are present in most organisms, and are known as TatA, TatB or TatE (9, 11) (Figure 1a). Translocation is initiated once a cargo protein with the correct signal peptide interacts with a docking complex composed of TatC and TatA-like proteins (38, 39). The specific interaction between the cargo protein and the docking complex has been implicated in substrate proofreading (11, 40-43), and the TatC component of the docking complex inserts the cargo proteins into the membrane (44). The cargo-docking complex then recruits the pore-forming TatA-like proteins and the proton-motive force is used as an energy source for translocation (40, 45, 46) (Figure 1b). Tat complexes of different sizes have been observed and are described in Table 1.

Phylogenetic studies of Tat systems have defined the composition of the principal Tat system as one TatC and two TatA-like components (47, 48). The replication of TatA or TatA-like proteins is probably a result of direct gene duplication as the gene for the second TatA-like component is often found elsewhere on the genome. In some cases these duplicated TatA-like proteins have diverged substantially from the 'original' TatA protein (48). This divergence is observed in a number of bacterial species and thylakoids where the second TatA-like proteins are termed TatB or Hcf106a, respectively. Though structurally similar, these diverged TatA-like proteins have specialized to a point where TatB explicitly associates with TatC in the docking complex and TatA assembles into the pore complex (49, 50).

The TatA-TatB divergence is not universal nor is the core system always defined as TatA, TatB and TatC, as demonstrated in staphylococci and B. subtilis. In staphylococci only one TatA protein is present (51, 52), and in B. subtilis the Tat system is composed of two parallel TatA-TatC pathways each with a single unique TatA (8). Similarly, in Corynebacterium glutamicum, which contains three TatA-like proteins (TatA, TatB, TatE), the essential components for translocation are TatA and TatC only (53). Hence, the optimum Tat system seems to be composed of three components (TatA, TatB and TatC), while the essential core could be defined as a binary TatA-TatC system.

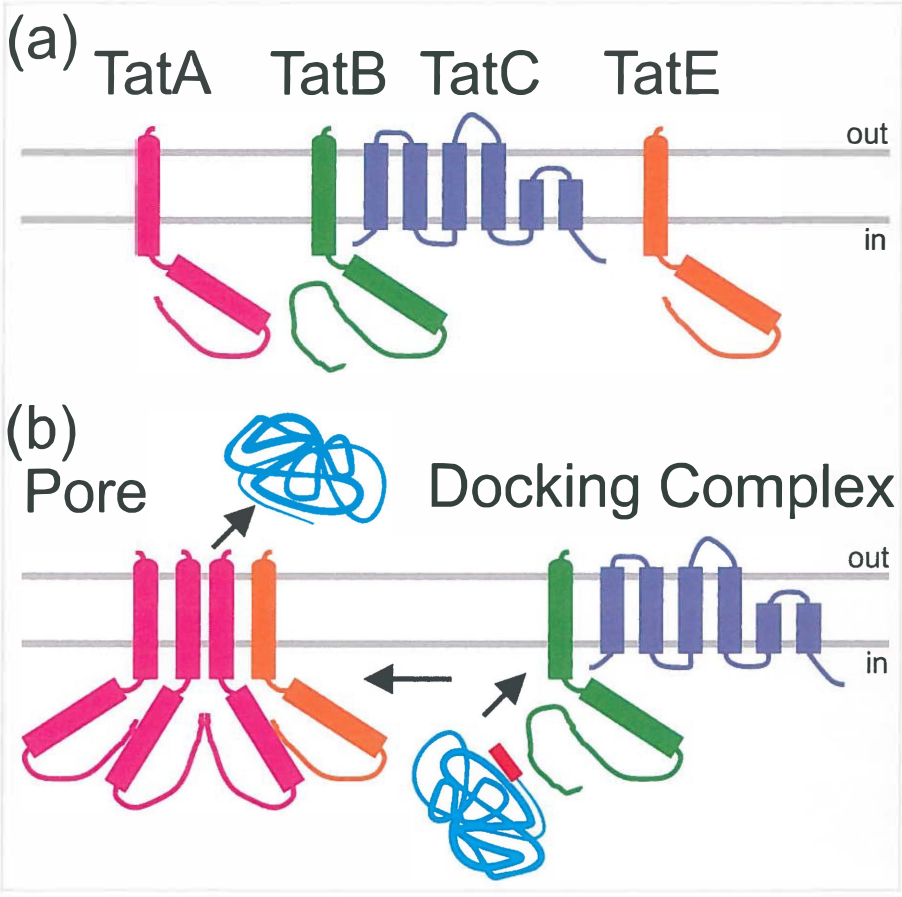

Figure 1: The components and consensus model for protein translocation via the Tat system. (a) The universally confirmed components of the Tat system are illustrated. The TatA-like proteins TatA, TatB and TatE are shown in pink, green and orange respectively, while the large transmembrane protein TatC is shown in purple. **(b)** Consensus model for protein translocation via the Tat system. The docking complex is composed of TatC and a TatA-like protein (often TatB). This docking complex forms the primary cargo-Tat interaction point. The Tat-dependent cargo protein in its 'translocation-approved' form interacts with the docking complex, where after pore-forming TatA-like proteins (TatA and/or TatE) are recruited. The pore adapts to the globular nature of the cargo, which is then translocated across the membrane and released from the Tat machinery.

Table 1. Tat complexes of *B. subtilis* and *E. coli* and their sizes.

E. coli			
TatA (11 kDa)	50-500 kDa (54-56)	TatAC	Aggregates and no clear complex (55)
TatB (18 kDa)	100-880 KDa (55) OR 100 kDa (57)	TatBC	440 KDa and 580 KDa (55, 56) OR 430 KDa (57) OR 370 KDa (54)
TatE (7kDa)	50-110 kDa (58)	TatAB	Aggregates with hints of a ladder (55) OR pools at the bottom of the gel (54)
TatC (30 kDa)	250 kDa (55) OR 220 kDa (57)	TatABC	440 KDa and 580 KDa (55) OR 430 KDa (57) OR 370 KDa (54)
B. subtilis			
TatAy (5 kDa)	Membrane: 200 kDa* (30) Cytoplasm: 5 MDa* (30)	TatAdCd	230 kDa (24)
TatAd (7 kDa)	Membrane: 270 KDa* (24, 59) OR 160 KDa* (24) OR 200 kDa* (his-tagged) Cytoplasm: 2 MDa* (24) Laddering described but size not discussed by Walther *et al* (60)	TatAyCy	200 kDa* (30)
TatAc (6 kDa)	100 kDa (59, 61)	TatAcCd	230 kDa (61)
TatCd (27 kDa)	66 kDa and 100kDa (61)	TatAcCy	200 kDa (61)
TatCy (28 kDa)	66 kDa (61)		

The predicted protein sizes according to gene products are indicated in brackets next to each protein. Complex sizes were mostly determined by blue-native gel electrophoresis, except for a few cases where gel filtration chromatography was used (indicated by *).

The TatC Protein

TatC is a protein with six membrane-spanning domains and highly conserved cytoplasmic loops (48, 62, 63). As mentioned above, TatC interacts with TatA-like proteins (often TatB) to form the docking complex and its role is to identify and verify cargo. A high degree of protein conservation between species is often a good indication of the importance of regions and residues within a protein. This is indeed the case in the first cytoplasmic loop of TatC, which directly interacts with cargo proteins (63-68). Furthermore, both cytoplasmic loops are directly associated with TatA (69). The transmembrane spanning domains of TatC interact directly with other TatC molecules (63, 70) as well as with the cargo (44, 67). Residues within the first two extracytoplasmic loops of TatC are not highly conserved between species. However, the secondary structure of these extracytoplasmic loops must be vital, as random mutagenesis studies have shown that these regions were specifically sensitive to substitutions (64, 71). Further, mutations in the C-terminal cytoplasmic region of *B. subtilis* TatCy block substrate translocation, implying this region is also important (68). Hence, structural and functional studies investigating TatC have shown a number of regions that directly interact with other components of the Tat system, and these regions have specific roles with regard to efficient Tat-dependent translocation.

Although duplications of TatA-like proteins are common, the Tat system in *B. subtilis* is distinctive in that there are two copies of *tatC* on the genome, *tatCd* and *TatCy* (48, 72). TatCd and TatCy each form complexes with TatAd or TatAy respectively, which operate in parallel as detailed in the following sections.

TatA and TatA-like Proteins

Sequence comparisons of TatA and TatA-like proteins have shown that variations have arisen due to gene duplications followed by further sequence divergence (48). In some cases the sequence divergence has resulted in proteins with specialized functions, which is particularly evident in *E. coli* and *Streptomyces coelicolor* where there are three *tatA*-like genes on the genomes. TatA and TatE are interchangeable and both form part of the pore during translocation (58, 73), while TatB functionality has specialized to form part of the docking complex (43, 44, 57, 74). *E. coli* TatA and TatB only share 20% sequence similarity (75). However, small mutations in TatA allow for complementation of a TatB mutant strain (76, 77) and a chimeric TatA-TatB protein is able to complement for the absence of both TatA and TatB (78). Further, *B. subtilis* TatAd is a bifunctional protein that is able to com-

pensate for the absence of TatA and TatB in *E. coli* (24). This underscores the view that, although TatA and TatB have evolved their own specific functions, they are structurally similar and with minor tweaking can replace one another.

TatA and TatA-like proteins are composed of a short N-terminal domain which sticks outward from the membrane, a single transmembrane domain, a short flexible hinge region and an amphipathic helix that is thought to lie flat against the membrane (79-81). The amphipathic helix region leads onto a second flexible end region containing a large number of densely charged residues (60). These densely charged residues have been suggested to form charged zippers that allow for the self-assembly of the TatA pore-complex. Most structural regions are involved in TatA functioning, and the hinge and amphipathic helix are particularly important (23, 24, 75, 78, 82-86). Only the C-terminal region was shown to be dispensable for TatB or TatA activity in *E. coli* (78).

Blue native gel electrophoresis of *E. coli* TatA has revealed a laddering of TatA complexes (Table 1) and electron microscopy studies have subsequently confirmed that *E. coli* TatA forms complexes with variations in size (22, 54). This has led to the theory that by changing the number of TatA components the pore size can adapt to the size of the substrate to be translocated. Though highly attractive, this theory is hard to reconcile with regard to the apparent lack of major size variations in complexes of *E. coli* TatE [58] or *B. subtilis* TatAd (59). Notably, the blue native laddering effect is greatly diminished for *E. coli* TatE and *B. subtilis* TatAd, and it remained so far undetected for *B. subtilis* TatAc (58-60). Nevertheless, *E. coli* TatE and *B. subtilis* TatAd and TatAc are able to compensate for TatA deficiency in *E. coli* (24, 58, 87). This raises the question how pore complexes composed of only *E. coli* TatE or *B. subtilis* TatA proteins accommodate cargo proteins of very different sizes while maintaining membrane integrity. Recent studies in *B. subtilis* have shown that TatAc can form a functional translocon (87) and that it assists TatAy in EfeB translocation (Chapter 3 of this thesis). It is therefore tempting to speculate that TatAc or a second TatA-like protein may have a role in pore size adaptability.

Quality control of Tat-dependent cargo proteins

The twin-arginine residues in the signal peptide region define the Tat system and its substrates. However, the signal peptide is not the only a necessary requirement for Tat-dependent translocation. The correct folding and cofactor incorporation of cargo proteins is vital in Tat systems. If the cargo pro-

tein is not correctly folded or if co-factors are inserted incorrectly, translocation is terminated and the cargo degraded (88-91). For example, it has been proposed that divalent metal ions lower down the Irving William series, such as Mn^{2+} and Fe^{2+}, are particularly important co-factors in Tat-dependent cargo proteins. These co-factors need to be inserted prior to translocation to ensure their stability and proper function (92). Strong evidence exists that the Tat machinery, and in particular the docking complex, is directly involved with Tat substrate quality control (17, 44, 74, 93). Further, if essential for survival, suppressor mutations can arise in the docking complex (TatC and TatB) to ensure translocation, as shown with the selectable reporter TEM-1 β-lactamase in several folded states (17).

The folding requirement of cargo proteins for translocation via Tat has been very clearly illustrated with proteins that need disulphide bonds for their stability and/or activity. This includes the heterologous single-chain Fv antibody fragments, heterodimeric F(AB) antibody fragments, human tissue plasminogen activator and *E. coli* TorA-PhoA fusion proteins (15, 16, 18, 94, 95). However, disulphide bond formation is not the only folding requirement for export of these proteins via Tat. When using the TorA-PhoA fusion protein as a model, translocation was hindered substantially when the protein was truncated while the region with disulphide bonds was not altered (18). Therefore, disulphide bond formation as well as correct co-factor insertion are important for Tat-dependent translocation. The importance of correct co-factor attachment and disulphide bond formation is addressed in Chapter 4 of this thesis with regard to the Tat-dependent membrane protein QcrA.

In the *E. coli* Tat system, certain cargo proteins have been associated with their own specialized chaperones and in some instances the signal peptide region of the cargo protein has been shown to directly interact with them. These chaperones allow for the incorporation of specific cofactors and the correct folding of the cargo proteins (91, 96). Such specific chaperone-substrate relationships include the hydrogenase-2 (NiFe) chaperoned by HybE (91) and TorA chaperoned by TorD (91). A chaperone function has also been proposed for DmsD in chaperoning the DmsA subunit of DMSO reductase (96), but a subsequent study showed that DmsD is completely dispensable for DmsA export (97).

In the case of Gram-positive bacteria no clear Tat-specific chaperones have as yet been identified. Nevertheless, Yeast two-hybrid (Y2H) studies performed with the *B. subtilis* Tat proteins against a library of proteins have suggested a number of Tat-interacting partners with potential chaperone or proofreading activity. For example, this study showed that WprA, an

extracytoplasmic cell wall-bound protease, is important for export of the Tat -dependent substrate EfeB (98). Further, the soluble chemoreceptor HemAT and the large transmembrane pentose transporter CsbC were shown to affect the amount of TatAd observed in the cell and, thus, to be important for export of the Tat-dependent substrate PhoD (98). Quantitative proteomic studies in *B. subtilis tat* mutant strains have also highlighted a number of proteins potentially associated with quality control. One of these, the iron-sulphur scaffold protein SufS, was found in decreased amounts in the cytoplasm of a *tat* mutant strain (total-*tat*) devoid of all Tat components (20). Furthermore, a chaperone associated with heat shock and iron-sulphur cluster assembly, DnaJ, was observed in higher amounts in the total-*tat* mutant strain (20, 99, 100). Although these proteomic approaches have as yet not confirmed any Gram-positive Tat-associated chaperones, they have put forward potential protein-protein partners and suggest a larger more complicated Tat-associated protein network than has previously been considered.

There has been some discussion in the field as to whether or not TatA molecules interact with the substrate in the cytoplasm before the cargo is inserted into the membrane. This would be an enticing theory, where TatA or TatA-like proteins interact with the cargo protein prior to the docking complex interactions. These discussions all revolve around identification and co-localization of TatA in the cytoplasm and contrasting results have been documented. Studies in *E. coli* with YFP-labelled TatA showed that TatA localized only at the membrane (101), while TatA labelled with ^{35}S-methionine showed both membrane and cytoplasmic localization (102). Analyses in *B. subtilis* with green fluorescent protein (GFP) labelled TatAc, TatAy and TatAd showed these proteins localized at the poles and membrane (103). However, other studies indicate that TatAd may associate with its substrate, pre-PhoD, in the cytoplasm and the membrane (69, 104, 105). When expression is induced in *E. coli,* TatAy (30) and TatAd (24) have also been observed in the cytoplasm as well as the membrane. Similarly, in *Streptomyces lividans,* TatA was shown to interact with TatB or its cargo in the cytoplasm (106, 107). Further studies in *S. lividans* showed that TatA or TatB complexes interact with the signal peptide region of Tat-dependent substrates in the cytoplasm (108). This led to theories where TatA-like proteins might have chaperone activities that lead/recruit substrates to the membrane translocation pore. An interesting additive is that, when the whole Tat system is hyper-expressed in *E. coli,* TatA forms tubes in the cytoplasm and the presence of these tubes is dependent on TatC (109). Under non-hyper-expressed conditions TatC also stabilizes and helps retain TatAd in the membrane (69). Cytoplasmic TatA may therefore have a broader role in the Tat translocation system. However, this may not reflect real physiological Tat-associated interac-

tions, since all these studies have been performed using induced Tat proteins with subsequent cell disruption and subcellular fractionation. This harsh treatment of the cells may well lead to a (partial) dissociation of TatA from the membrane, which cannot be readily distinguished from the possible presence of soluble TatA that is in the process of being inserted into the membrane.

Altogether, there are multiple proteins and quality control checkpoints associated with correct translocation of Tat substrates. These include protein interactions between substrate specific chaperones, the signal peptide region of the substrate and the docking complex, to ensure correct folding of substrates and co-factor insertion. Many ongoing research efforts are aimed at the molecular dissection of these checkpoints, and this is particularly necessary for an improved understanding of mechanisms determining Tat pathway function in Gram-positive bacteria.

Crosstalk between secretion pathways

Cellular systems are complex and proteins can often have overlapping functions. The core Tat systems has its select components, however proteins associated with it allow for crosstalk with other cellular systems. These systems can include other secretion systems, translation systems as well as post -translocational interactions.

Crosstalk between the Sec and Tat secretion systems has been shown in *Streptomyces*. Here, overproduction of the Tat system had a direct effect on the amount of Tat-dependently and Sec-dependently secreted substrates, as there was an increase in Tat-dependent secretion and a decrease in Sec-dependent secretion (110). Furthermore, the deletion of *tatB* increased Sec-dependent secretion in *S. lividans* (111). Collaboration between translocation systems has been documented in *E. coli* expressing an unusual Rieske iron-sulphur protein from *S. coelicolor*. A portion of this normally Tat-dependent cargo protein was shown to use a Sec-YidC-dependent membrane insertion mechanism while the remaining portion was translocated Tat-dependently (21).

Further convergence between the Tat and Sec systems relates to signal peptide processing. Signal peptide processing is necessary for the release of Sec-translocated proteins from the membrane (14, 112). Signal peptide processing of Tat-dependent cargo precursors in *E. coli* (113) and in *B. subtilis* (20, 72) has been observed. The Rieske-iron protein QcrA has widespread Tat-dependency (20, 114-116). Intriguingly, the QcrA protein of *B.*

subtilis is receptive for signal peptidase processing, resulting in the release of a smaller form into the growth medium (20). This processing and subsequent release of the extracytoplasmic QcrA domain is hard to reconcile with the function of QcrA in electron transfer, which suggests a potential sheddase function of signal peptidases (112). The view that the *B. subtilis* signal peptidase can act as sheddases is supported by the previous observation that certain polytopic membrane proteins can also be cleft by these enzymes (117, 118).

Another example of Sec and Tat pathway crosstalk is associated with the cell wall-bound and extracellular quality control protease WprA, which is known to degrade Sec-dependently translocated proteins at the membrane-cell wall interface (119, 120). The afore-mentioned Y2H studies have shown direct protein-protein interactions between TatAy-TatCy and WprA (98). Accordingly, a *wprA* mutation resulted in a decreased amount of the TatAy-TatCy-dependently translocated EfeB and YkuE proteins, suggesting a functional WprA-TatAy-TatCy-EfeB/YkuE interaction (98). Further, studies in *B. subtilis* have shown that multiple extracytoplasmic proteases are involved in degrading EfeB (121). Unexpectedly, in strains lacking these extracellular proteases the need for WprA in EfeB biogenesis was suppressed suggesting that WprA is somehow needed to protect EfeB against the activity of these other proteases (121).

Specific Gram-positive bacterial Tat systems

Gram-positive bacteria were originally defined by the results of the Gram stain. The properties of Gram-positive bacteria are so that their thick peptidoglycan layer retains the Gram stain and with it the purple-blue colour. Although quite rudimental the Gram stain does successfully describe and delineate fundamental features of the bacterial envelope. Nonetheless, it is important to mention that within the Gram-positive phylogenetic tree there is a subdivision of bacteria that, though testing positive in the traditional Gram staining, contain a different cellular envelope structure. Instead of a single membrane and a cell wall, the envelope of these bacteria includes a peptidoglycan-mycolic acid wall structure and notably an extra membrane (Figure 2a). It is therefore more accurate to define them as diderms (35, 122-124). Bacteria that have this extra membrane include mycobacteria, corynebacteria, rhodococci and nocardiae. Hence, for the purpose of this review the bacterial groups detailed below are defined as monoderm and diderm Gram-positive bacteria.

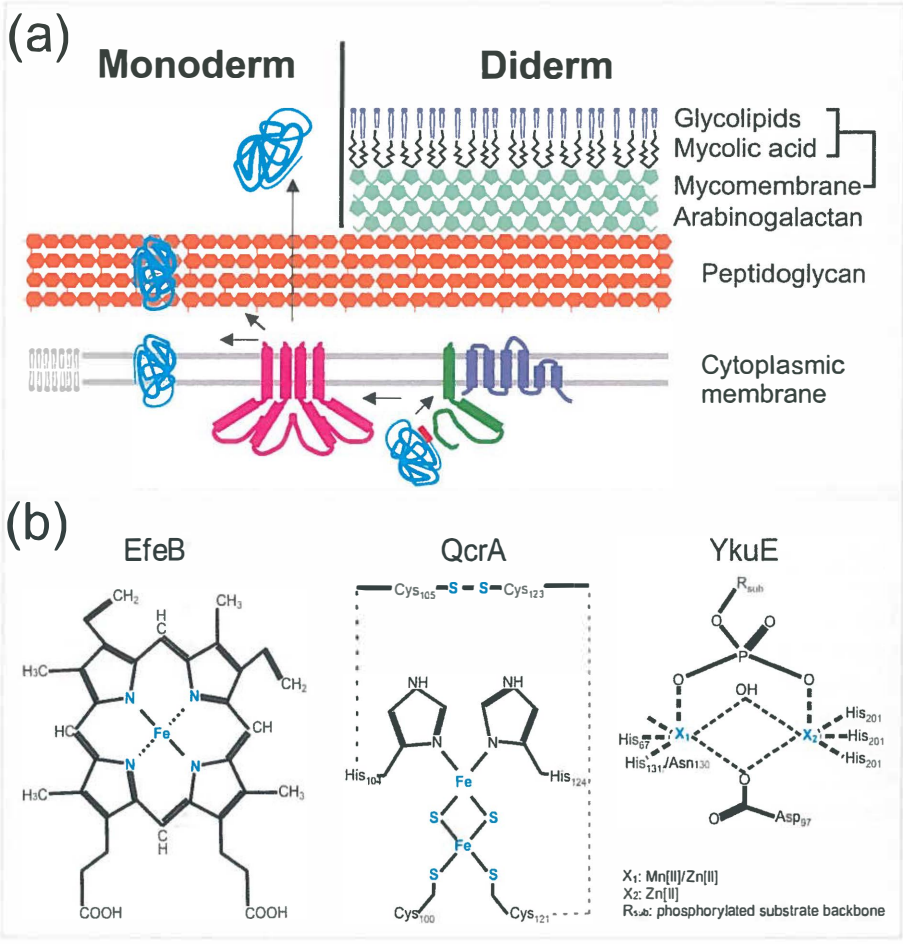

Figure 2: A representation of bacterial cell envelope structure and co-factors of known Tat-dependent cargo. (a) An illustration of the envelope structure of diderm and monoderm Gram-positive bacteria. **(b)** The co-factors of known *B. subtilis* Tat-dependent cargo. The three TatAy-TatCy dependent cargo proteins each have known co-factors: EfeB (YwbN) has a heme group (125), QcrA has a Rieske-iron sulphur co-factor and contains a disulphide bond (126-128), and YkuE has a metal co-factor (129).

Monoderm Gram-positive bacterial Tat systems

Bacillus subtilis

B. subtilis is the predominant model organism for Gram-positive bacteria. It has a GRAS status, is highly amenable to genetic engineering, and is able to produce large titres of secreted proteins (12-14). Within the genus *Bacillus* there are other industrially relevant workhorses, such as *Bacillus licheniformis* and *Bacillus amyloliquefaciens,* but also pathogenic species such as *Bacillus anthracis* and *Bacillus cereus.* This genus is therefore of interest for fundamental scientific research, medical microbiological aspects, and applications in bio-industrial settings (13).

As mentioned before in this review, the *B. subtilis* Tat machinery is composed only of TatA and TatC proteins. There are three TatA components in *B. subtilis*: TatAc, TatAd and TatAy. Together with TatC, two of these TatA components combine to form two parallel TatA-TatC pathways, namely TatAy-TatCy and TatAd-TatCd. The TatAy-TatCy and TatAd-TatCd pairs are expressed operonically (130-132), and each pathway works independently and has its own substrate specificities (20, 72, 132). Studies have shown that TatAd-TatCd is expressed only under conditions of low phosphate, which corresponds to the expression of its one known substrate PhoD (72, 131-133). In contrast, TatAy-TatCy is expressed consistently over many tested conditions and has a broader range of native substrates including EfeB (YwbN), QcrA, YkuE and potentially a few more (14, 20, 72, 129). The three known TatAy-TatCy-dependent cargo proteins have known co-factors (Figure 2b), and in the case of QcrA it not only has a Rieske Iron-sulphur 2Fe-2S cofactor, but also a known disulphide bond essential for correct folding (126-128).

The TatAd-TatCd pathway shows more flexibility with regard to substrate specificity, as when overexpressed, TatAd-TatCd is able to translocate the normally TatAy-TatCy-dependent substrate EfeB (134). Reversed, however, TatAy-TatCy is not able to translocate the TatAd-TatCd dependent PhoD (134). Inter-species inter-pathway variation is observed when the *B. subtilis* pathways are expressed in *E. coli,* or when cargo proteins with *E. coli* signal peptides are expressed in *B. subtilis.* Both *B. subtilis* pathways are able to translocate GFP with Tat-dependent *E. coli* signal peptides when the pathways are expressed in *E. coli.* However, when the same cargo proteins were expressed in *B. subtilis,* translocation was not exclusively Tat-dependent (24, 30, 135). This suggests that, although the *B. subtilis* Tat pathway is technically able to translocate these cargo proteins, other mechanisms in *B. subtilis* itself may prevent the Tat-dependent translocation of these heterologous GFP fusion proteins (24, 30, 135). This underscores the view that the Tat-associated

quality control and proofreading mechanisms in *E. coli* differ from those of *B. subtilis*.

The role of the third *B. subtilis* TatA component, TatAc, has until recently remained ambiguous. It is consistently expressed (131), but *tatAc* mutant strains have as yet not shown any phenotypes (14, 25, 72). Nor could TatAc compensate for the absence of TatAy or TatAd in translocation of EfeB or PhoD, respectively (134). However, when expressed in *E. coli,* complexes of TatAc-TatCd or TatAc-TatCy were fully functional and translocated the *E. coli* cargo proteins TorA, AmiC and AmiA (61). Furthermore, when expressed in *E. coli*, TatAc was also able to complement for the absence of *E. coli* TatA and TatB (59). Protein-protein interaction studies with Y2H techniques have shown that TatAc may not only interact directly with itself, with TatAd and TatAy, but also with the 'haem-based aerotactic transducer' HemAT (98). Importantly, HemAT was also shown to be important for secretion of PhoD under low phosphate conditions. Therefore, the observed Y2H interaction with HemAT is biologically relevant (98). Importantly, recent studies described in Chapter 3 of this thesis have uncovered that TatAc can assist TatAy with regard to active EfeB translocation. These findings suggest that TatAc in *B. subtilis* might represent a protein with an intermediate TatA-TatB function.

In *B. subtilis* various phenotypes associated with Tat-deficiencies have been observed. These phenotypes may be due to the absence of specific cargo proteins. However they could also suggest relationships associated with quality control, compensatory mechanisms or even crosstalk between systems. Therefore, these phenotypes may imply direct or indirect responses. Quantitative proteomic studies showed that a number of biofilm-associated proteins were found in decreased amounts in *tatAy-tatCy* deficient strains. This led to the observation that a deficiency in *tatAy-tatCy* results in a delayed biofilm formation phenotype at the liquid-air interface (20). However a direct link between this delayed biofilm phenotype and the Tat system is not yet clear and probably relates to an indirect response. Phenotypes directly related to the absence of the dependent cargo proteins have also been described. In particular, EfeB has a ferrous iron scavenging function and forms part of the membrane associated EfeUOB iron transporter (125). Consistent with its role in iron scavenging, the absence of active EfeB in *tatAy-tatCy* deficient strains results in a lowered growth rate under limited iron availability (136). Interestingly this EfeB-iron-associated phenotype is amplified in Lysogeny broth (LB) without salt (i.e. NaCl), where active EfeB becomes essential for normal growth (136). EfeB is part of the *efeUOB* operon, however, it is also independently controlled by cell envelope stress factors (125, 131). The specificity of EfeB to scavenge ferrous iron has implica-

tions under microaerobic conditions, where ferrous iron is more abundant than ferric iron, giving strains producing EfeB a growth advantage. Notably, the acquisition of ferrous iron involves the H_2O_2-dependent oxidation of ferrous iron to ferric iron by EfeB, thereby effectively eliminating potentially detrimental reactive oxygen species at membrane-cell wall interface (125).

Apart from the EfeB-associated response, studies have suggested that the salinity of the environment in which *B. subtilis* finds itself has direct implications with regard to the Tat-dependency of other substrates. In LB medium with high (6%) NaCl concentrations some ectopically expressed Tat -dependent cargo proteins showed independence to the Tat system (135, 136). Conversely, in LB without NaCl, *B. subtilis* strains expressing the *B. cereus* or *Staphylococcus aureus* TatA-TatC translocases showed increased Tat-dependent secretion of ectopically expressed EfeB-myc (83). Although intriguing, exactly why and how environmental salinity changes the dependency of cargo proteins on Tat is presently not clear. However, some data suggest that TatA-TatC translocases of Gram-positive bacteria are intrinsically sensitive to salt, which would be indicative of mechanistically relevant electrostatic interactions within the Tat translocon (60, 83).

Streptomyces

Streptomycetes are mycelial bacteria normally found in the soil. However, due to their ability to secrete large amounts of proteins they have become workhorses in various industrial applications, particularly regarding the production of antibiotics (137). Studies on the Tat system of *Streptomyces coelicolor, Streptomyces scabies* and *Streptomyces lividans* have shown that, unlike all other bacterial systems, the *Streptomyces* Tat system is a major secretion pathway in terms of the numbers of cargo proteins (138-140).

The essential Tat system in *Streptomycetes* is composed of one TatC and two TatA-like proteins with specialized TatA and TatB functions (141, 142). Studies have indicated that both TatA and TatB associate with Tat-dependent pre-proteins in the cytoplasm (107, 142). This has led to theories of TatA-like cytoplasmic chaperone activity, as discussed in the previous section.

The *Streptomyces* Tat system can be engineered in various ways. By simply inducing expression of tatABC, the secretion of the Tat-dependent substrate xylanase C was increased (110). Also, the induced production of the phage shock protein PspA improves Tat-dependent protein secretion in both *S. lividans* and *E. coli* (143, 144). Although the exact role and mechanism of how PspA assists the Tat system is not clear, PspA binds phospholipids

(145) and direct interactions between PspA and the N-terminal domain of *E. coli* TatA have been shown (146). In both *E. coli* and *Streptomyces*, PspA is induced and shown to be important under conditions of extracytoplasmic stress (147, 148). It has been suggested that PspA suppresses proton leakage (145). Therefore its role may be associated with membrane integrity and helping the cell to maintain the proton-motive force during protein translocation via Tat.

Staphylococcus

The *staphylococcal* Tat system is composed of a single TatA and TatC. The Tat machinery is not present in all staphylococcal species. However, tatA and tatC have been identified on the genomes of *S. aureus, S. carnosus*, and *Staphylococcus haemolyticus* (51). The *staphylococcal* Tat system is functional in S. aureus and S. carnosus and is able to translocate a number of non-heterologous proteins (LipA, Spa and GFP) (51, 149). Not many staphylococcal Tat substrates have been identified, and two-dimensional gel electrophoresis of the exoproteome of tat mutated *staphylococci* showed that it did not differ from that of the wild-type strains (51, 52). The sole known staphylococcal Tat-dependent substrate is the iron-dependent peroxidase FepB (51). Genomic association initially identified this as a potential Tat-dependent cargo protein, as the *febABC* operon is found downstream of the *tatA-tatC* genes (51). The products of the *febABC* operon are highly reminiscent of the EfeUOB iron uptake system in B. subtilis. Further FepB and B. subtilis EfeB have 40% sequence similarity, both have peroxidase activity and share the same ferrous iron scavenging function (51, 125).

Diderm Gram-positive bacterial Tat systems

Diderm Gram-positive bacteria have an extra outer-membrane, and cargo proteins are translocated via the Tat system into the inter-membrane space between the cytoplasmic membrane and the outer mycomembrane. It is not clear exactly how the cargo proteins of this Tat system are subsequently secreted into the extracellular milieu and whether secondary secretory mechanisms, similar to those in Gram-negative bacteria, are involved in this process. For example, porins form major integral proteins in the mycomembrane of *C. glutamicum* and are thought to contribute to the cell envelope permeability of these diderm Gram-positive bacteria (123, 150, 151).

Corynebacterium

Corynebacteria are aerobic or facultative anaerobic non-motile bacteria that have functional Tat systems. This genus includes pathogens such as *Corynebacterium diphtheria*e and *Corynebacterium jeikeium*. On the other hand, non -pathogenic species such as *C. glutamicum* have been widely used in industry (152). The Tat system in *Corynebacterium* is generally composed of two TatA-like proteins and a TatC. In some instances, such as *C. glutamicum* and *Corynebacterium efficiens*, the gene for a third TatA-like protein, TatE, is also present (153-155). Although there are three TatA-like proteins in *C. glutamicum*, the essential components for translocation are TatA and TatC only. Notably, the expression of all TatA-like proteins does increase maximum protein secretion (53).

The Tat system in *C. glutamicum* is particularly efficient in translocating heterologous proteins. Industrially relevant co-factor containing proteins, such as carbohydrate oxidases, are secreted in their active form by the Tat system in *C. glutamicum* (31), and enzymes such as isomalthohydrolase, glutaminase and transglutaminase are secreted successfully via Tat to titres of >100mg/l (28, 53). Further, when comparing the translocation of GFP in three different bacterial industrial workhorses - *C. glutamicum, B. subtilis* and *S. carnosus* – only *C. glutamicum* secreted properly folded fluorescent GFP (149).

The overall production of industrially relevant enzymes has been improved by the overproduction of Tat components, in particular TatC (29). Also the efficiency of secretion can be manipulated by replacing the signal peptide of the cargo proteins, as significant improvements in translocation were observed when the native *C. glutamicum* signal peptide was replaced with a *B. subtilis* signal peptide or a *E. coli* TorA signal peptide (53, 149). Hence using the *C. glutamicum* Tat system in industrial settings for the biotechnological production of relevant enzymes and products is becoming more and more feasible and a promising strategy.

Mycobacterium

The genus *Mycobacterium* includes a number of notorious pathogenic species such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*. *M. tuberculosis* is of particular relevance as it has a functional Tat system, and in 2011 alone, 8.7 million new cases of tuberculosis and 1.4 million deaths were reported (156). In mycobacteria the Tat system is composed of TatA, TatB and TatC (157). It follows the similar duplication trend observed in other three-component Tat systems, where the core system is composed of a

tatC-tatA-like operon with the gene of the third component, *tatB*, found elsewhere on the genome (48, 158). The Tat system in *M. tuberculosis* is unique, especially within the Gram-positive bacteria, in that it is one of the few examples where the Tat system is essential for survival of the organism (9, 158, 159).

A total of 18 *M. tuberculosis* proteins with functional Tat signal peptides have been confirmed using a β-lactamase reporter system in the related *Mycobacterium smegmatis* (160, 161). Of these 18 known substrates, a number are known to contribute directly to virulence and drug resistance, such as the β-lactamase BlaC and the phospholipase C enzymes PlcA and PlcB (160, 162). The Tat system is absent from humans and combining this with the knowledge that the system is essential in *M. tuberculosis*, has made the Tat system an alluring drug target for future treatment of tuberculosis patients (163).

Introduction to the Bdb system

The main pathway for protein secretion is the Sec pathway (1-4, 7). This pathway translocates proteins in unfolded or loosely folded states. Upon membrane passage via Sec, the translocated proteins need to be processed and correctly folded. In the case of particular proteins the formation of disulphide bonds, that is the oxidation of two cysteine thiol groups, is fundamental for correct folding and stabilization of the folded state. The oxidative reaction necessary for disulphide bond formation can occur spontaneously. However, disulphide bond formation between the correct cysteine residues is usually catalysed *in vivo* by specific enzymes, namely thiol-disulphide oxidoreductases (TDORs) (5, 6). These TDORs are characterized by an active site containing the sequence Cys-X-X-Cys. Furthermore, they often contain an overall thioredoxin fold made up of a five-stranded β-sheet and four α-helices (6, 164). Depending on the cellular location and redox potential of an individual TDOR's active site, these proteins can act as thiol oxidases (forming disulphide bonds), disulphide reductases (breaking disulphide bonds) or as isomerases (moving disulphide bonds) (164, 165).

In *E. coli* the TDOR pathways have been studied in great detail, and the pathway associated with extracytoplasmic disulphide bond formation is aptly referred to as the disulphide bond pathway (Dsb) (5, 166). The periplasmic DsbA protein is the major catalyst for disulphide bond formation. This protein relays electrons from its Sec-secreted substrates onto the inner membrane protein DsbB. This DsbA-DsbB redox pairing of pro-

teins, where proteins form an electron relay team, is conserved throughout all known oxidative protein folding pathways (5, 166, 167).

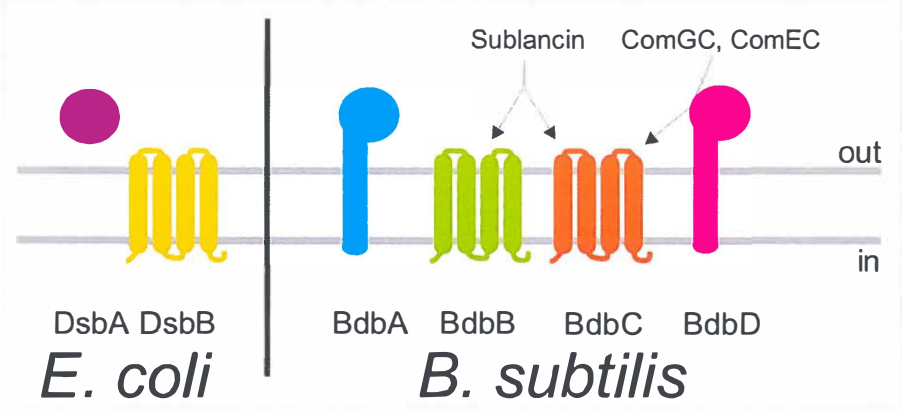

Figure 3. The extracytoplasmic TDORs involved in post-translocational disulphide bond formation. The *E. coli* DsbA-DsbB system is shown on the left, while the Bdb system of *B. subtilis* is shown on the right. Known *B. subtilis* Bdb substrates are shown and the manners in which they are oxidized by the Bdb system are indicated by arrows.

A number of TDOR proteins have been identified in *B. subtilis*. These TDORs are involved in both disulphide reductase (thioredoxin, ResA, ResB, CcdA) and thiol oxidase activities. More specifically in *Bacillus* the Dsb-like thiol oxidases involved in extracytoplasmic disulphide bond formation are referred to as *Bacillus* disulphide bond proteins (Bdb) (5, 168). Four Bdb proteins have been identified in *B. subtilis*, namely BdbA, B, C and D. The genes for these proteins are grouped in pairs on the genome: *bdbA* and *bdbB* are found in the Spβ prophage region, while *bdbC* and *bdbD* form an operon on the core genome (169). The role of BdbA is not clear as it is dispensable for the folding of known Bdb substrates and lacks compensatory roles with regards to known Bdb interactions (170). BdbB and BdbC are associated with the correct folding of the Spβ prophage-encoded bacteriocin sublancin 168 (170). BdbC and BdbD form a redox pair important for oxidative folding of the competence proteins ComGC (171) and ComEC (172). BdbC and BdbD are also needed for the heterologous secretion of the *E. coli* alkaline phosphatase PhoA (168, 169). Apart from the competence- and bacteriocin-associated proteins no other native Bdb substrates have been identified in *B. subtilis*. Intriguingly however, BdbC and BdbD do have a role in the oxidative misfolding of apocytochrome *c* thereby precluding the proper insertion of haem (173, 174). In wild-type cells, this oxidative misfolding is prevented by

the action of the reductive CcdA-ResA pathway, which ensures that apocytochrome *c* remains in a reduced state until haem is bound (175).

The previous studies have suggested that BdbC and BdbD make up the primary oxidative TDOR pair in *B. subtilis* and, consistent with this view, the *bdbC* and *bdbD* genes are expressed throughout the cell cycle under a wide range of physiologically and industrially relevant conditions (131). Importantly, the expression of *bdbC* and *bdbD* is not specific in cells that are competent for genetic transformation suggesting the possible existence of BdbC and BdbD substrates that are not associated with competence. Despite extensive molecular biological and proteomics analyses on cell wall or spent culture media, no such substrates have as yet been identified in *B. subtilis* (7, 176). This suggested that particular membrane proteins might be substrates for oxidative folding by BdbC and BdbD. In Chapter 5 of this thesis, this theory was further investigated by analysis of the membrane proteome of strains devoid of BdbC-BdbD.

Scope of this thesis

The research performed towards this thesis covered the Tat system for translocating pre-folded proteins across the cytoplasmic membrane of *B. subtilis* and the Bdb system associated with the folding of translocated proteins as described in the introductory **Chapter 1**.

Studies documented in **Chapter 2** described studies which used mass spectrometry techniques, in particularly quantitative proteomics, to investigate strains deficient in either one or all Tat-associated pathways of *B. subtilis*. To this end, cells were fractionated and the cytoplasmic, membrane and extracellular proteome fractions were investigated. Changes in the amount or the presence of specific proteins were considered indicative of a Tat relationship. The observed changes were potentially due direct interactions with the Tat system, or to indirect ramifications of strains devoid of Tat components. The final outcome of this study was that a plethora of novel potentially Tat-associated proteins were identified, a slow biofilm formation phenotype was uncovered, and the Rieske iron-sulphur protein QcrA was confirmed as a *B. subtilis* Tat-dependent cargo protein.

Chapter 3 deals with the active translocation of the TatAy-TatCy-dependent cargo protein EfeB (YwbN). Previous studies had shown that EfeB is strictly dependent on TatAy-TatCy for translocation, and that *efeB* or *tatAy -tatCy* mutants grown in media without NaCl display a severe lysis phenotype due to EfeB deficiency. For the studies described in **Chapter 3**, ad-

vantage was taken of this lysis phenotype as a readout for EfeB translocation by various TatAy or TatCy mutant proteins constructed by site-directed mutagenesis. Importantly, growth experiments in LB without NaCl with strains lacking different chromosomal *tat* genes, but expressing particular site-specific TatAy mutant proteins, allowed for a grading of the severity of EfeB translocation defects. This led to the finding that TatAc assists TatAy in the translocation of EfeB. Thus, the studies uncovered a biological function for the enigmatic TatAc protein of *B. subtilis.* Importantly, this function suggests that TatAc represents an evolutionary intermediate between the TatA-like proteins TatA and TatB that have been described in other bacteria, such as *E. coli*.

In **Chapter 4**, export of the Tat-dependent cargo protein first confirmed in **Chapter 1**, namely QcrA, is described in detail. The ability of site-specifically mutated QcrA proteins to be fully translocated was investigated with special focus on amino acid residues involved in co-factor insertion or disulphide bond formation. Indeed, the Tat-associated translocation of QcrA mutant proteins affected in co-factor binding or disulphide bond formation was blocked. This implies that both folding and co-factor insertion is essential for the correct translocation of this Tat-dependent substrate. Interestingly, a proofreading hierarchy between the different QcrA mutants was observed. Cargo proteins unable to complete the disulphide bond-assisted folding were degraded immediately, while co-factor-associated mutant proteins were partially processed and reached the cytoplasm-membrane interface before translocation was halted. Importantly, this is the first example of a native Tat-dependent protein showing a requirement for both co-factor binding and disulphide bond formation in translocation. Further studies described in **Chapter 4,** addressed the questions how QcrA translocation is affected by site-specific amino acid mutations in TatAy or TatCy, and whether the NaCl concentration in the growth medium influences Tat-dependent QcrA translocation. The results show that the Tat system is not affected by environmental salinity with regard to its ability to translocate QcrA. Furthermore, when comparing the translocation profile of QcrA, slight variations were observed as compared to those described in **Chapter 3** for EfeB. These findings suggest that residues in the amphipathic helix of TatAy are involved in substrate-specific interactions.

For the studies described in **Chapter 5**, mass spectrometry techniques were once again used. However, unlike in **Chapter 1** where research was focused on the Tat system, the studies in **Chapter 5** investigated the membrane proteome of strains devoid of Bdb proteins. The *B. subtilis* extracellular milieu is highly proteolytic and proteins not correctly folded by the Bdb proteins would be rapidly degraded. The membrane fractions of Bdb

devoid strains were examined and the presence or absence of proteins was considered indicative of the involvement of this system for post-translocational protein folding. In doing so, a number of novel Bdb-affected proteins were observed. In particular, the salt stress-associated protein ProA was absent from the *bdbC-bdbD* mutant strain. Consistent with the absence of ProA, an osmotic shock-sensitive phenotype was uncovered in the *bdbC-bdbD* mutant strain.

Finally, **Chapter 6** gives the reader an overview of the results presented in this thesis and places them in a broader context.

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Chapter 2

Novel twin-arginine translocation pathway-dependent phenotypes of *Bacillus subtilis* unveiled by quantitative proteomics

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Abstract

The Twin-arginine Translocation (Tat) pathway is known to translocate fully folded proteins across bacterial, archaeal and organellar membranes. To date, the mechanisms involved in processing, proofreading and quality control of Tat substrates have remained largely elusive. *Bacillus subtilis* is an industrially relevant Gram-positive model bacterium. The Tat pathway in *B. subtilis* differs from that of other well-studied organisms in that it is composed of two complexes operating in parallel. To obtain a better understanding of this pathway in *B. subtilis* and to identify Tat-associated proteins, the *B. subtilis* 'Tat proteome' was investigated by quantitative proteomics. Metabolically labeled proteins from cytoplasmic, membrane and extracellular fractions were analyzed by LC-MS/MS. Changes in the amounts of identified peptides allowed for quantitative comparisons of their abundance in *tat* mutant strains. The observed differences were suggestive of indirect or direct protein-protein relationships. The rich data set generated was then approached in hypothesis-driving and hypothesis-driven manners. The hypothesis-driving approach led to the identification of a novel delayed biofilm phenotype of certain *tat* mutant strains, whereas the hypothesis-driven approach identified the membrane protein QcrA as a new Tat substrate of *B. subtilis*. Thus, our quantitative proteomics analyses have unveiled novel Tat pathway-dependent phenotypes in *Bacillus*.

Introduction

The twin-arginine translocation (Tat) pathway is a protein secretion pathway found in bacteria, archaea and the thylakoid membranes of chloroplasts (9-11). In prokaryotes, it is the only known pathway able to translocate fully folded proteins, including proteins with bound cofactors. Thus, the proteins *exported* via the Tat pathway can have very diverse functions, ranging from basic processes like anaerobic respiration in *Escherichia coli* to virulence in pathogenic *Pseudomonas* species. Additionally, the Tat pathway has attracted attention for potentially valuable applications in biotechnology (12, 15, 177).

The Tat pathway is well characterized in *E. coli, Streptomyces* and chloroplasts and numerous Tat-translocated substrates have been identified in these organisms (178). In contrast, very few Tat-translocated substrates have been recognized in the model Gram-positive bacterium *Bacillus subtilis* (7, 25). Furthermore, the Tat pathway in *B. subtilis* differs from the Tat pathways studied in other bacteria. Instead of a single complex composed of

three subunits named TatA, TatB and TatC as present in *E. coli*, TatB is absent from *B. subtilis* and active Tat complexes are merely composed of TatA and TatC subunits (8, 72). Moreover, there are two Tat complexes with partially overlapping specificities active in *B. subtilis*, which are known as TatAyCy and TatAdCd. The operons encoding each of these complexes are found at different locations on the genome and are expressed under different conditions (72, 131, 132). Additionally there is a third TatA protein, TatAc, which was recently shown to form active Tat translocases with the TatCy and TatCd proteins when expressed in *E. coli* (87).

The translocation of fully folded proteins is one of the hallmarks of the Tat pathway. It has been shown that the correct folding and cofactor attachment of substrate proteins is critical for efficient translocation via Tat (15, 17, 74). However, relatively little is known as to potential chaperones and partners involved in processing, proofreading and quality control in this pathway (10, 91, 179). A second important characteristic in Tat-translocation is the presence of a twin-arginine (RR) motif in the N-region of the substrates' signal peptides. Early low-stringency bioinformatic analyses of the *B. subtilis* proteome predicted 69 proteins with signal peptides containing potential RR-motifs (25). However to date only four Tat substrates have been confirmed experimentally. The TatAdCd pathway is needed for the secretion of PhoD, a phosphodiesterase, under conditions of phosphate starvation (132). The TatAyCy pathway exports the Dyp-type peroxidase YwbN, which has been implicated in the acquisition of iron (72, 136), and the wall-bound metallo-phosphoesterase YkuE (129). The fourth substrate is the secreted esterase LipA, which becomes Tat-dependent only when its high-level overexpression is induced (180). The Tat pathway is not essential under standard laboratory growth conditions (25), but growth and viability of *tatAyCy* mutant strains is severely affected upon iron-limitation (136). Consistent with the important role of iron in many processes, the *tatAyCy* genes are known to be expressed under all conditions tested and they are upregulated upon iron limitation (131, 181).

Intriguingly, it was so far not known how the Tat pathway influences the composition of the different subproteomes of the *B. subtilis* cell, which proteins it may interact with, or the wider physiological roles it may play in the cell. This relates to the fact that investigations into Tat proteins, their interacting partners and substrates have been partly hindered by the hydrophobic nature of these membrane proteins. However, recent developments in shotgun mass spectrometry and metabolic labelling approaches have revolutionised the analysis of membrane proteomes (182-184). Notably, information regarding the presence or absence of an assortment of proteins can be suggestive of direct protein-protein interactions as well as indirect rela-

tionships. The present studies were therefore aimed at detecting changes in the relative amounts of proteins present in the different subproteomes of *B. subtilis tat* mutant strains. To this end, cells were metabolically labelled with $^{14}N/^{15}N$ and individual proteins were identified and quantified. Since changes in the amounts of particular proteins observed are suggestive of direct or indirect responses to the absence of the Tat machinery, the results of this shotgun approach could indicate novel Tat-dependent protein relationships. Conventional molecular microbiological techniques were subsequently used to validate these results. This led to the identification of QcrA as a TatAyCy-dependent membrane protein. Furthermore, a delayed biofilm formation phenotype of *tatAyCy* mutant strains was uncovered.

Experimental Section

Bacterial strains, plasmids, media and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Lysogeny Broth (LB) was composed of 1 % tryptone, 0.5 % yeast extract and 1 % NaCl. Unless otherwise specified, bacteria were grown in LB broth at 37 °C under vigorous shaking, or on LB agar plates incubated at 37 °C. When appropriate the cultures were supplemented with antibiotics: *E. coli* cultures with 100 µg/ml ampicillin (Ap) and *B. subtilis* cultures with 2 µg/ml erythromycin (Em), 5 µg/ml chloramphenicol (Cm), 10 µg/ml tetracycline (Tc), 100 µg/ml spectinomycin (Sp), or 20 µg/ml kanamycin.

The correct insertion of pXTC plasmids in the chromosomal *amyE* gene was confirmed by the absence of amylase activity on LB plates supplemented with 1 % starch. Expression of *ywbN* inserted into pXTC was induced by the addition of 1-2 % xylose 2 hours prior to protein extraction. *B. subtilis* cells were grown to competence in Paris Medium (PM) (168). DNA used for transformation was extracted from *B. subtilis* BR151 SL6628, *B. subtilis* 168 *tatAdCd*, *B. subtilis* 168 *tatAyCy*, or *B. subtilis* 168 total-*tat*.

Table 1. Strains and plasmids used in this study

Strains	Relevant properties	Ref
B. subtilis		
168	*trpC2*	(185)
168 *tatAdCd*	*trpC2, tatAd-tatCd*::Km^r	(25)
168 *tatAyCy*	*trpC2, tatAy-tatCy*::Sp^r	(72)
168 *total-tat$_2$*	*trpC2, tatAd-tatCd*::Km^r, *tatAy-tatCy*::Sp^r; *tatAc*::Em^r	(72)
168 X-*ywbN*	*trpC2, amyE*::*xylA-ywbN*-c-*myc*	(25)
168 *tatAdCd* X-*ywbN*	*trpC2, tatAd-tatCd*::Km^r; *amyE*::*xylA-ywbN*-c-*myc*	(25)
168 *tatAyCy* X-*ywbN*	*trpC2, tatAy-tatCy*::Sp^r; *amyE*::*xylA-ywbN*-c-*myc*	(25)
168 *total-tat$_2$* X-*ywbN*	*trpC2, tatAd-tatCd*::Km^r, *tatAy-tatCy*::Sp^r; *tatAc*::Em^r, *amyE*::*xylA-ywbN*-c-*myc*	(72)
168 *qcrA*	*trpC2, qcrA*::pPP435	This study
BLR151 SL6628	*trpC2 lys3 metB10 qcrA*::pPP435	(186)
3610	Strain NCBI3610, Undomesticated wild strain	(187)
3610 *tatAdCd*	NCBI 3610 transformed with chromosomal DNA 168 ΔtatAdCd, *tatAd-tatCd*::Km^r	This study
3610 *tatAyCy*	NCBI 3610 transformed with chromosomal DNA 168 ΔtatAyCy, *tatAy-tatCy*::Sp^r	This study
3610 *total-tat*	NCBI 3610 transformed consecutively with chromosomal DNA 168: *tatAd-tatCd*::Km^r, *tatAy-tatCy*::Sp^r; *tatAc*::Tc^r	This study
168 *sipSUVW*	*trpC2, tyr, his,nic, ura, rib, met, ade, rib$^+$, sipS, sipU, sipV, sipW*, Em^r, Tc^r	(188)
168 *sipTUVW*	*trpC2, tyr, his, nic, ura, rib, met, ade, sipT, sipU, sipV, sipW, Cm^r, Sp^r, Tc^r*	(188)
Plasmids		
pHB-*AdCd*	pHB201 vector carrying the *tatAd-tatCd* operon; Cm^r, Em^r	(180)
pHB-*AyCy*	pHB201-derivative containing the *tatAy-tatCy* operon; Em^r; Cm^r	(136)
pHBQcrAwt	pHB201-derivative containing *qcrA*; Em^r; Cm^r	This study

Metabolic labelling, protein sample preparation and Mass Spectrometry (MS)

B.subtilis cells were grown aerobically at 37 °C in the presence of 1 % xylose in a synthetic minimal medium (189) supplemented with either ^{15}N-ammonium sulphate/^{15}N- L-tryptophan (0.078 mM, 98 atom % excess, Cambridge Isotope Laboratories, Andover, USA) or ^{14}N-ammonium sulphate and ^{14}N-L-tryptophan. For the analysis of membrane and cytosolic fractions, cells were harvested by centrifugation at an optical density of 600 nm (OD_{600}) of 1. ^{14}N-labeled cells of each sample were prepared in duplicate and ^{15}N cells were pooled to obtain a labelled pooled standard according to MacCoss *et al* (190). The washed cells of the labelled ^{15}N pool and the unlabelled samples were disrupted mechanically in a French press (Simaminco SLM, New York, USA) and the cell debris was removed via centrifugation (14000 x g at 4 °C for 20 min). Subsequently, the protein concentration of the unlabelled ^{14}N samples and the pooled ^{15}N samples was determined. After that, the same protein amount of the ^{14}N and ^{15}N pooled protein extracts were mixed to obtain a metabolically labelled cell extract for further fractionation procedures. Membrane proteins were enriched according to the protocol published in Eymann *et al* (191) omitting the extraction of proteins by n-dodecyl-β-D-maltoside treatment. The supernatant after the first ultracentrifugation step in the membrane enrichment protocol represented the cytosolic fraction.

The extracellular proteome was prepared according to Antelmann *et al* (192). Cells were separated from the growth medium by centrifugation. Subsequently, proteins in the medium were precipitated by adding 10 % trichloroacetic acid (TCA) and overnight incubation on ice. The precipitate was washed thoroughly with ethanol and the resulting protein pellet was solved in urea/thiourea. After determination of the protein concentration and pooling of the ^{15}N-labeled extracellular proteins, equal protein amounts of the ^{14}N samples and ^{15}N-pooled samples were mixed to obtain the metabolically labelled extracellular fractions.

All samples were analysed by the GeLC-MS workflow. After electrophoretic fractionation of each mixed sample by one-dimensional SDS-PAGE, gel lanes were sliced into 10 equidistant gel pieces followed by tryptic digestion as described by Eymann *et al* (191). The resulting peptides were first concentrated and desalted on a trapping column (Waters nanoACQUITY UPLC column, Symmetry C18, 5 µm, 180 µm x 20 mm, Waters Corporation, Milford, Mass., USA) for 3 min at a flow rate of 1 ml/min with 99 % buffer A (0.1 % acetic acid). Subsequently the peptides were eluted and subjected to reversed phase column chromatography (Waters BEH 1.7 µm, 100-µm i. D. x 100 mm, Waters Corporation, Milford, Mass., USA) operated on a

nanoACQUITY-UPLC (Waters Corporation, Milford, Mass., USA). The peptides were separated with a non-linear 80-min gradient from 5–60 % ACN in 0.1 % acetic acid at a constant flow rate of 400 nl/min. MS and MS/MS data were acquired using a 7-T Finnigan LTQ-FT mass spectrometer (Thermo Fisher, Bremen, Germany) equipped with a nanoelectrospray ion source. After a survey scan in the FT-ICR (r= 50,000) MS/MS data were recorded for the five most intensive precursor ions in the linear ion trap. Singly charged ions were not taken into account for MS/MS analysis. The *.dta files extracted from *.raw files using BioworksBrowser 3.3.1 SP1 (Thermo Fisher Scientific) without charge state deconvolution and deisotoping were searched with SEQUEST version v28 (rev.12) (Thermo Fisher Scientific) against a *B. subtilis* target-decoy protein sequence database (complete proteome set of *B. subtilis* extracted from UniprotKB release 12.7 (193) with a set of common laboratory contaminants) compiled with BioworksBrowser. The searches were done in two iterations: First, for the GeLC-MS analyses the following search parameters were used: enzyme type, trypsin (KR); peptide tolerance, 10 ppm; tolerance for fragment ions, 1 amu; b- and y-ion series; variable modification, methionine (15.99 Da); a maximum of three modifications per peptide was allowed. In the second iteration the mass shift of all amino acids completely labelled with ^{15}N nitrogen was taken into account in the search parameters. Resulting *.dta and *.out files were assembled and filtered using DTASelect (version 2.0.25) (parameters GeLCMS: -y 2 -c 2 -C 4 --here --decoy Reverse_ -p 2 -t 2 -u --MC 2 -i 0.3 --fp 0.005). Protein identification data were validated with an in-house java script to ensure that each protein hit relies on at least two different peptides as judged by the amino acid sequences.

The validated search results served as the base for the software Census (194) to obtain quantitative data of 14N peaks (sample) and 15N peaks (pooled reference (190)). Peptide ratios were determined by Census and subsequently exported (R^2 values bigger than 0.7 and only unique peptides; proteins failing to be relatively quantified were checked manually in the graphical user interface for on/off proteins). Proteins relatively quantified with at least 2 peptides were taken into account for the subsequent analysis. The MS data associated with this manuscript may be downloaded from the ProteomeCommons.org Tranche network using the following hash keys:

hash#1:0GJpEIgc9XRXfoQRfAzSEkEBGHqygWCbtlbwKx0fFqfGfQEICgow+E42MSdwKDHUL1XdIrQWaPy89DA7a4rxRKbs5NEAAAAAAAABFHg==

hash#2:xsTAKFJ5exwpludpHY1rlOeg1TsaF8jmeNk2FurqV9sfgavdMdKO0c+qb680jJiwsxU+65MavoNMwRCTv2dIgeM7WXYAAAAAAABDHA==

hash#3:PytCDarVmPAyPGFAcgunavFLUG4rsVKQantKkmUP7ZaZiU/NZ7E6VmWioJmjm1mE53cKMo/deB4EKk6kNlXbpdDztGYAAAAAAABPBA==

These hash keys may be used to prove exactly what files were published as part of this manuscript's dataset, and the hash may also be used to check that the data has not changed since publication. Furthermore, the proteomics data are summarized in Supplementary Tables S1 and S2.

Biofilm Assays

The floating pellicle assay was adapted from Branda *et al* (195). Overnight cultures in LB broth were used to inoculate minimal MSgg medium for biofilm formation. MSgg medium contained 0.5 % glycerol, 0.5 % potassium glutamate, 5 mM K_2HPO_4, 50 µg/ml phenylalanine, 2 µM thiamine, 50 µg/ml Tryptophan, 50 µM $FeCl_3$, 100 mM MOPS, 700 µM $CaCl_2$, 2mM $MgCl_2$, 50 µM $MnCl_2$, 1 µM $ZnCl_2$. Cultures were left for 10 days at 24 °C until film-like pellicle structures formed on the liquid-air interface.

Whole-cell protein extractions

Whole-cell protein extractions were performed after cultures were grown to an OD_{600} of 3.5 – 4. Culture aliquots (1.2 ml) were treated with 170 µl Complete protease inhibitor (Roche) and centrifuged. The growth medium fraction was removed and proteins in this fraction were precipitated with TCA. The cells were resuspended in 120 µl LDS gel loading buffer and reducing agent (NuPAGE, Invitrogen). Cells were disrupted by bead-beating three times with glass beads at 6500 rpm for 3 s with 30 sec intervals (Precellys 24 lysis & homogenization, Bertin Technologies). Samples were stored at -20 °C.

Subcellular fractionation

For subcellular fractionation studies, cultures were grown to mid-exponential phase and cells were harvested by centrifugation at 4000 rpm at 4 °C. The growth medium fraction was removed and proteins in 1 ml growth medium were precipitated with TCA. The cell fraction was resuspended in protoplast buffer (100 mM Tris, 20 mM $MgCl_2$, 20 % sucrose, 1 mg/ml lysozyme, 0.01 % DNase, Complete protease inhibitor [Roche]) and incubated at 37 °C for 30 min. The resulting protoplasts were then separated from liberated cell wall proteins by centrifugation at 4000 rpm at 4 °C. The cell wall fraction was collected and the protoplasts were resuspended in disruption buffer (50 mM Tris pH 8.0, 2.5 mM EDTA). The protoplasts were disrupted by bead beating three times at 6500 for 30 sec with 30 sec pauses (Precellys 24 lysis & homogenization, Bertin Technologies). The cytoplasmic content and membranes were separated from the glass beads by aspiration after spinning down the beads at 10,000 rpm. The sample was centrifuged at 200,000 g and the cytoplasmic (supernatant) fraction was removed where after the membrane pellet was resuspended in solubilisation buffer (20 mM Tris, 10

% glycerol, 50 mM NaCl, 0.03 % DDM) and left at 4 °C. Lastly, non-solubilized membranes were pelleted by centrifugation at 100,000 g and the supernatant with solubilized membrane proteins was collected. Samples were stored at -20 °C.

Gel Electrophoresis and Western Blotting

Proteins were separated using NuPAGE gels (Invitrogen) and transferred onto nitrocellulose membranes (Protan, Schleicher&Schuell) by semi-dry blotting. The QcrA, TrxA, and BdbD proteins were detected with polyclonal antibodies raised in rabbits (Eurogentec, Belgium). Bound antibodies were then further detected by fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit from LiCor Biosciences) and visualized at 700 and 800 nm with the Odyssey Infrared Imaging System (LiCor Biosciences).

Results

Quantitative comparisons of subcellular proteomes of tat mutant strains by metabolic labeling and LC-MS/MS.

In the present studies, quantitative proteomics analyses of the cytoplasmic, membrane and extracellular fractions of various *tat* mutant *B. subtilis* strains and their parental strain 168 were performed by metabolic labeling. To this end, all strains were grown in duplicate in defined minimal medium with either ^{14}N or ^{15}N. Specifically, the ^{15}N-labeled strains included a *tatAyCy* mutant, a *tatAdCd* mutant, a mutant lacking all *tat* genes and the parental strain 168, all of which ectopically expressed the *ywbN* gene from a xylose-inducible promoter. Furthermore, the parental 168 reference strain not expressing *ywbN* was labeled with ^{14}N. Upon harvesting, the ^{14}N-labeled reference strain was combined with the individual ^{15}N-labeled strains before cells were fractionated, proteins extracted and LC-MS/MS analyses performed as originally proposed by MacCoss *et al* (190). As all ^{15}N-labeled strains were compared to the ^{14}N-labeled parental strain, we were able to directly compare the peptide abundances in individual *tat* mutant strains with each other. At least two unique peptides were required to confirm the presence of a protein.

In total, 908, 453 and 447 proteins were identified in the cytoplasmic, membrane and extracellular fractions, respectively. The coverage of quantifiable peptides ranged between 88-97 % in the cytoplasmic, 80-90 % in the membrane, and 70-90 % in the extracellular fractions. These rich data

sets, detailed in supplementary materials Table S1, were processed in two different, hypothesis-driving and –driven manners.

Subtle changes in the subproteomes of B. subtilis tat mutants.

For the hypothesis-driving data processing, the ratios of peptides labelled with ^{14}N or ^{15}N were calculated and compared (Tables 2-4). The data was processed such that the ratios were only compared when generated in each biological replicate and the variation between the biological replicates was consistent (p-value <0.05). These constraints drastically decreased the pool of potential leads, but increased the validity of the targets identified. Differences between the quantified peptides were considered significant if the fold changes between the background and the *tat* mutant strains were more than 1.5. This cut-off may appear low, but it has been shown to generate biologically relevant results in other studies (196). The targeted locations of proteins were predicted by combining information from 6 different transmembrane prediction programs (197-202) and three different secretion prediction programs (203-205). Importantly, the Tat pathway was not essential under the conditions tested and no change in growth phenotype was observed (data not shown). Hence, subtle changes observed in this study could generate innovative theories concerning potential Tat pathway functions.

It should be noted that all the ^{15}N-labeled strains also ectopically expressed YwbN. Several mild changes related to YwbN expression were observed upon comparison of the ^{15}N-labeled peptides from the YwbN-producing parental strain 168 and the ^{14}N-labeled peptides of the 168 reference strain not expressing YwbN (Supplementary Table S2). The rationale for expressing YwbN was that this is a known Tat substrate (72), which might have served as a positive control for Tat-dependent secretion. However, YwbN was not quantifiably or consistently identified, which probably relates to limitations in the detection by LC-MS/MS. Nevertheless, the YwbN expression by itself did not detract from the general utility of our data, because YwbN expression was induced in all ^{15}N-labelled strains, allowing the separation of YwbN-related changes listed in Table S2 from the Tat-related effects presented in Tables 2-4.

Table 2. Hypothesis-driving results of quantitative proteomic analyses of cytoplasmic fractions

Cytoplasm Fold Increase

	WT	*AdCd*	*AyCy*	total- *tat*	Targeted localization	Function
DnaJ	D	**1.63**	D	**1.58**	Cytoplasmic/ not specified	Chaperone protein DnaJ
YddK	D	**2.08**	**2.30**	**2.09**	Cytoplasmic/ not specified	Uncharacterized protein
YfkO	D	**5.49**	D	**4.97**	Membrane	Putative NAD(P)H nitroreductase

Cytoplasm Fold Decrease

	WT	*AdCd*	*AyCy*	total- *tat*	Targeted localization	Function
AhpC	-1.48	D	-1.42	**-2.56**	Cytoplasmic/ not specified	Alkyl hydroperoxide reductase subunit
AhpF	-1.42	**-2.70**	-1.42	D	Membrane	Alkyl hydroperoxide reductase
CheY	D	**-1.78**	D	-	Cytoplasmic/ not specified	Chemotaxis protein CheY homolog
SufS	-1.21	**-1.44**	-1.20	**-1.52**	Cytoplasmic/ not specified	Probable cysteine desulfurase
FtsE	D	**-2.04**	-	D	Membrane	Cell division ATP-binding protein FtsE
KatA	D	**-4.14**	D	**-3.75**	Cytoplasmic/ not specified	Vegetative catalase
MetE	-1.28	**-2.09**	-1.26	**-2.14**	Cytoplasmic/ not specified	Cobalamin-independent methionine synthase
MetK	-1.25	D	D	**-1.57**	Cytoplasmic/ not specified	Methionine adenosyltransferase
MtnW	D	**-1.59**	-1.39	**-1.63**	Cytoplasmic/ not specified	RuBisCO-like protein
ThrS	D	**-2.26**	D	**-2.07**	Cytoplasmic/ not specified	Threonyl-tRNA synthetase 1
AdcA	-1.44	-	**-1.59**	D	Membrane/ Secreted	Probable zinc transport system zinc-binding
YitJ	-1.23	**-1.92**	-1.26	**-1.88**	Cytoplasmic/ not specified	S-methylmethionine:homocysteine methyltransferase
YoaC	D	**-2.29**	D	D	Cytoplasmic/ not specified	Putative sugar kinase
YoaD	-1.20	D	-1.20	**-2.00**	Cytoplasmic/ not specified	Putative 2-hydroxyacid dehydrogenase
YxjH	D	-1.45	D	**-1.58**	Cytoplasmic/ not specified	Uncharacterized protein
XylA	Not in ^{14}N	-	-	-	Cytoplasmic/ not specified	Xylose isomerase
YtbE	Not in ^{14}N	Not in ^{15}N	-	-	Cytoplasmic/ not specified	Uncharacterized oxidoreductase
YwbN	Not in ^{14}N	-	Not in ^{14}N	-	Secreted	Putative peroxidase

Fold changes represent the differences between the ^{14}N-labeled 168 strain and the four ^{15}N-labeled strains. A fold change of more than 1.5 was considered significant and is indicated in bold. The predicted localization of the protein and its predicted function are indicated. '-': Not Detected, D: Deteced, Not in ^{14}N: Not Detected in the ^{14}N-labeled 168 reference strain, Not in ^{15}N: not detected in ^{15}N-labeled strain

Table 3. Hypothesis-driving results of quantitative proteomic analyses of membrane fractions

Membrane Fold Increase

	WT	*AdCd*	*AyCy*	total- *tat*	Targeted localizations	Function
AtpD	D	D	1.36	**1.79**	Cytoplasmic/ not specified	ATP synthase subunit beta
ClpX	D	D	-	**1.60**	Cytoplasmic/ not specified	ATP-dependent Clp protease
GltA	D	D	**1.51**	D	Membrane	Glutamate synthase
IlvH	D	D	**1.73**	-	Cytoplasmic/ not specified	Acetolactate synthase small subunit
OpuAA	D	D	**2.11**	**1.98**	Membrane	Glycine betaine transport ATP-binding protein
PurL	D	D	D	**1.64**	Cytoplasmic/ not specified	Phosphoribosylformylglycinamidine synthase
QcrB	D	**1.60**	D	-	Membrane	Menaquinol-cytochrome c reductase
RplB	D	D	1.25	**1.53**	Cytoplasmic/ not specified	50S ribosomal protein
RpoB	D	D	1.28	**1.61**	Cytoplasmic/ not specified	DNA-directed RNA polymerase subunit beta
RpoC	D	D	D	**1.56**	Cytoplasmic/ not specified	DNA-directed RNA polymerase subunit beta
RpsB	1.39	D	1.48	**2.03**	Cytoplasmic/ not specified	30S ribosomal protein S2
RpsK	D	D	D	**2.20**	Cytoplasmic/ not specified	30S ribosomal protein S11
SdpC	D	**1.60**	D	D	Membrane	Killing factor sdpC
SerA	D	D	**1.56**	D	Cytoplasmic/ not specified	D-3-phosphoglycerate dehydrogenase
Tuf	D	1.41	**1.52**	**2.04**	Membrane	Elongation factor Tu
YdjF	D	D	-	**2.07**	Membrane	Phage shock protein A homolog
YjlD	D	D	1.18	**1.66**	Membrane	NADH dehydrogenase-like protein
YomK	D	D	**1.50**	**1.70**	Membrane	SPBc2 prophage-derived uncharacterized protein

Membrane Fold Decrease

	WT	*AdCd*	*AyCy*	total- *tat*	Targeted localizations	Function
ComGA	D	**-2.01**	D	**-1.83**	Membrane	ComG operon protein
SrfAB	-1.47	**-1.78**	D	D	Cytoplasmic/ not specified	Surfactin synthase subunit 2
YhbJ	-1.18	**-1.53**	-1.42	D	Membrane	Putative efflux system component
YoaB	-1.12	**-1.77**	-1.22	**-1.83**	Membrane	Putative transporter
YpuA	-1.22	-1.18	**-1.56**	D	Membrane/ Secreted	Uncharacterized protein
YvrC	D	D	**-1.87**	D	Membrane/ Secreted	Uncharacterized ABC transporter substrate-binding lipoprotein
SunT	D	D	D	**-1.74**	Membrane	Sublancin-168-processing and transport ATP-binding protein
TlpB	D	D	D	**-1.69**	Membrane	Methyl-accepting chemotaxis protein
PbpH	D	-1.06	D	**-1.53**	Membrane	Penicillin-binding protein H

Fold changes represent the differences between the ^{14}N-labeled 168 strain and the four ^{15}N-labeled strains. A fold change of more than 1.5 was considered significant and is indicated in bold. The predicted localization of the protein and its predicted function are indicated. '-': Not Detected, D: Deteced, Not in ^{14}N: Not Detected in the ^{14}N-labeled 168 reference strain, Not in ^{15}N: not detected in ^{15}N-labeled strain

Table 4. Hypothesis-driving results of quantitative proteomic analyses of growth medium fractions.

	Growth Medium Fold Increase					
	WT	***AdCd***	***AyCy***	**total-*tat***	**Targeted localization**	**Function**
AhpC	1.41	D	**1.82**	D	Cytoplasmic/ not specified	Alkyl hydroperoxide reductase subunit C
AroH	1.32	D	D	**1.61**	Cytoplasmic/ not specified	Chorismate mutase
GapA	1.45	**2.01**	**2.34**	**1.98**	Cytoplasmic/ not specified	Glyceraldehyde-3-phosphate dehydrogenase 1
FusA	D	**2.56**	**3.22**	D	Cytoplasmic/ not specified	Elongation factor G
BdhA	1.49	D	D	**1.57**	Cytoplasmic/ not specified	(R,R)-butanediol dehydrogenase
PurS	1.23	D	1.49	**1.52**	Cytoplasmic/ not specified	Phosphoribosylformylglycinamidine synthase
YpjP	0.85	D	D	**1.77**	Membrane/ Secreted	Uncharacterized protein
LtaS2	D	**1.72**	D	**1.65**	Membrane	Polyglycerol phosphate synthase 2
Tpx	1.37	D	**1.95**	D	Cytoplasmic/ not specified	Probable thiol peroxidase
AroA	D	D	**1.89**	D	Cytoplasmic/ not specified	Protein AroA(G)
Adk	D	D	**1.75**	D	Cytoplasmic/ not specified	Adenylate kinase (AK)
AtpC	D	**1.87**	D	D	Cytoplasmic/ not specified	ATP synthase epsilon chain
CheY	D	D	**1.75**	D	Cytoplasmic/ not specified	Chemotaxis protein CheY homolog
Crh	D	-	**1.68**	D	Cytoplasmic/ not specified	HPr-like protein
DapA	D	1.44	**1.56**	1.40	Cytoplasmic/ not specified	Dihydrodipicolinate synthase
HupA	1.12	**1.62**	1.13	D	Cytoplasmic/ not specified	DNA-binding protein HU 1
FbaA	1.25	**1.62**	D	D	Cytoplasmic/ not specified	Probable fructose-bisphosphate aldolase
GcvH	D	D	**1.64**	-	Cytoplasmic/ not specified	Glycine cleavage system H protein
LeuD	D	D	**1.93**	D	Cytoplasmic/ not specified	3-isopropylmalate dehydratase small subunit
Mpr	D	**2.41**	D	-	Membrane/ Secreted	Extracellular metalloprotease
PtsH	D	**2.36**	**1.52**	D	Cytoplasmic/ not specified	Phosphocarrier protein HPr
RpsF	D	**1.51**	1.25	1.20	Cytoplasmic/ not specified	30S ribosomal protein S6
YdbP	D	**1.93**	1.39	1.41	Cytoplasmic/ not specified	Thioredoxin-like protein
YheA	D	**2.16**	D	D	Cytoplasmic/ not specified	Uncharacterized protein
YqeY	D	**1.62**	1.20	D	Cytoplasmic/ not specified	Uncharacterized protein
YugI	D	**1.62**	1.28	D	Cytoplasmic/ not specified	General stress protein 13
SufD	D	D	**1.76**	-	Cytoplasmic/ not specified	FeS cluster assembly protein
LutC	D	-	**2.10**	D	Cytoplasmic/ not specified	Lactate utilization protein C
IlvK	D	-	**1.95**	D	Cytoplasmic/ not specified	Branched-chain-amino-acid aminotransferase 2
YodC	D	D	**1.83**	D	Cytoplasmic/ not specified	Putative NAD(P)H nitroreductase
TrxA	D	D	**1.54**	D	Cytoplasmic/ not specified	Thioredoxin (Trx)
RsbT	D	-	**1.74**	D	Cytoplasmic/ not specified	Serine/threonine-protein kinase

Table 4. Continued: Hypothesis-driving results of quantitative proteomic analyses of growth medium fractions.

	WT	*AdCd*	*AyCy*	total- *tat*	Targeted localization	Function
Growth Medium Fold Increase continued...						
SerA	D	D	**1.83**	D	Cytoplasmic/ not specified	D-3-phosphoglycerate dehydrogenase
Pyk	D	1.17	**1.92**	D	Cytoplasmic/ not specified	Pyruvate kinase
YorD	1.38	D	**1.54**	D	Cytoplasmic/ not specified	SPBc2 prophage-derived stress response protein
MetE	D	D	**1.69**	-	Cytoplasmic/ not specified	Cobalamin-independent methionine synthase
Pta.	D	D	**1.78**	D	Cytoplasmic/ not specified	Phosphate acetyltransferase
Growth Medium Fold Decrease						
LytA	D	D	**-1.72**	D	Membrane/ Secreted	Membrane-bound protein
LytB	D	D	**-12.63**	D	Membrane/ Secreted	Amidase enhancer (Modifier protein of major autolysin)
LytF	D	**-1.64**	D	D	Membrane/ Secreted	Endopeptidase lytF
OpuBC	D	**-1.67**	D	D	Membrane/ Secreted	Choline-binding protein
Pel	D	**-2.10**	D	D	secreted	Pectate lyase
PelB	D	**-1.76**	D	D	Membrane/ Secreted	Pectin lyase
PelC	-1.39	**-1.60**	D	D	secreted	Pectate lyase C
PonA	-1.49	**-1.83**	**-2.13**	D	Membrane/ Secreted	Penicillin-binding protein 1A/1B
RplA	D	D	**-3.36**	-	Cytoplasmic/ not specified	50S ribosomal protein L1 (BL1)
RplE	D	D	**-2.11**	-	Cytoplasmic/ not specified	50S ribosomal protein L5 (BL6)
RplN	D	**-3.37**	**-3.76**	D	Cytoplasmic/ not specified	50S ribosomal protein L14
RpmE2	D	Not in ^{15}N	**-8.29**	Not in ^{15}N	Cytoplasmic/ not specified	50S ribosomal protein L31 type B
RpsE	D	D	**-4.29**	D	Cytoplasmic/ not specified	30S ribosomal protein S5 (BS5)
RpsP	D	**-2.91**	D	D	Cytoplasmic/ not specified	30S ribosomal protein S16 (BS17)
SdpC	D	**-1.81**	D	D	Membrane	Killing factor sdpC
Upp	-1.36	**-1.58**	**-1.53**	D	Cytoplasmic/ not specified	Uracil phosphoribosyltransferase
XlyA	D	**-2.17**	D	**-2.24**	Cytoplasmic/ not specified	N-acetylmuramoyl-L-alanine amidase
XynA	D	**-1.96**	**-1.82**	D	secreted	Endo-1,4-beta-xylanase A (Xylanase A)
XynC	D	D	**-3.17**	D	Membrane/ Secreted	Glucuronoxylanase (Endoxylanase xynC)
YbdN	-1.18	**-1.87**	D	**-2.32**	secreted	Uncharacterized protein
YbdO	D	**-1.54**	D	D	Cytoplasmic/ not specified	Uncharacterized protein
YbxI	D	D	**-5.86**	D	Membrane/ Secreted	Probable beta-lactamase
YclQ	D	**-1.63**	D	D	Membrane/ Secreted	ABC transporter solute-binding protein
YkwD	D	**-1.88**	D	D	Membrane/ Secreted	Putative lipoprotein

Table 4. Continued: Hypothesis-driving results of quantitative proteomic analyses of growth medium fractions.

Growth Medium Fold Decrease continued...

	WT	*AdCd*	*AyCy*	total-*tat*	Targeted localization	Function
YitM	D	**-1.72**	D	**-1.57**	Membrane	Uncharacterized protein
YkoJ	D	-0.87	**-2.78**	D	secreted	Uncharacterized protein
YkwD	D	D	**-2.17**	D	secreted	Uncharacterized protein
YlxG	D	**-1.58**	D	-	Cytoplasmic/ not specified	FlaA locus uncharacterized protein
YoaJ	D	**-1.81**	**-3.04**	D	Membrane/ Secreted	Expansin
YoaW	-1.10	**-1.63**	D	**-1.56**	Membrane/ Secreted	Uncharacterized protein, associated with biofilm
YqgA	D	**-3.71**	**-6.85**	**-4.58**	Membrane/ Secreted	Uncharacterized protein
YqgS	D	D	**-1.67**	-	Membrane	Uncharacterized protein
YraI	D	D	**-3.46**	D	Membrane	Uncharacterized protein
YrpE	D	-	**-3.44**	D	Membrane/ Secreted	Uncharacterized protein
YueB	D	D	**-1.94**	-1.48	Membrane	Bacteriophage SPP1 adsorption protein
AprE	D	-1.43	**-1.64**	-1.23	Membrane/ Secreted	Subtilisin E
YwbM	D	**-1.59**	D	D	secreted	Uncharacterized protein
YxaL	D	**-1.58**	D	-1.43	Membrane/ Secreted	Uncharacterized protein
YxkC	-1.29	**-2.03**	D	D	Membrane	Uncharacterized protein
YvbJ	D	**-2.08**	D	-1.38	Membrane/ Secreted	Uncharacterized protein
YvyC	-1.14	**-1.86**	D	**-1.52**	Cytoplasmic/ not specified	Uncharacterized protein
YpuD	D	-	**-2.34**	-	Membrane/ Secreted	Uncharacterized protein
YolB	D	**-1.78**	D	D	Cytoplasmic/ not specified	Spβ prophage-derived uncharacterized protein
LipA	D	D	**-1.70**	-1.20	Membrane/ Secreted	Lipoyl synthase
LipB	-1.48	D	**-1.93**	D	Membrane/ Secreted	Extracellular esterase (lipase B)
TasA	-1.29	**-1.80**	**-1.81**	**-1.50**	Membrane/ Secreted	Spore coat-associated protein N
Csn	D	**-4.04**	**-4.82**	D	Membrane/ Secreted	Chitosanase
Epr	D	**-2.53**	D	**-1.72**	Membrane/ Secreted	Minor extracellular protease
FlhP	-1.10	**-1.92**	D	-0.82	Cytoplasmic/ not specified	Flagellar hook-basal body complex protein
FliD	D	**-1.79**	D	**-1.52**	Cytoplasmic/ not specified	Flagellar hook-associated protein 2
FliK	D	**-2.54**	D	D	Membrane	Probable flagellar hook-length control protein

Fold changes represent the differences between the ^{14}N-labeled 168 strain and the four ^{15}N-labeled strains. A fold change of more than 1.5 was considered significant and is indicated in bold. The predicted localization of the protein and its predicted function are indicated. '-': Not Detected, D: Deteced, Not in ^{14}N: Not Detected in the ^{14}N-labeled 168 reference strain, Not in ^{15}N: not detected in ^{15}N-labeled strain

Of the 908 proteins identified in the cytoplasm, 18 proteins with diverse functions in metabolism and oxidative stress management were found to be present in significantly different amounts when comparing the reference and *tat* mutant strains (Table 2). As the proteins with fold changes observed here were associated with the cytoplasm and did not have signal peptide regions, these potential leads suggested possible interacting partners or Tat-deficiency-related compensatory pathways. In the membrane fraction, 27 of the 453 identified proteins, were differentially present in at least one of the strains (Table 3). Based on their localization, these 27 proteins may represent potential substrates or interaction partners. However, they may also reflect compensatory mechanisms due to the absence of particular Tat proteins. In the extracellular fraction, 447 proteins were identified of which 89 showed significant fold changes between the *tat* mutant strains and the parental strain (Table 4). In this case not only proteins with predicted signal peptides were identified at different levels, but also proteins that are known to be primarily located in the cytoplasm, the membrane- or the cell wall

A number of extracellular proteins, which are known or suggested to be involved in motility and biofilm formation (i.e. TasA, YoaW, Epr, LytF and LytA/B (206-208)) showed significant fold changes. This led us to investigate the motility and biofilm-forming abilities of *B. subtilis tat* mutant strains. *B. subtilis* is able to form various biofilm morphologies in the laboratory, including floating pellicles where a layer of biofilm forms at the liquid-air interface of stationary cultures (209). As no motility phenotype was observed, we focused on possible effects of *tat* mutations on biofilm formation. Notably, *B. subtilis* 168 is not considered an ideal model to study biofilm formation (195, 210). Nonetheless, pellicle biofilm formation at the liquid-air interface was observed for *B. subtilis* 168 (Figure 1). As expected, these pellicles were formed slower and remained substantially thinner than observed for the undomesticated *B. subtilis* strain 3610. A clear biofilm phenotype related to the deletion of the *tatAy-tatCy* genes was observed in *B. subtilis* 168. As shown in Figure 1a, the pellicle formation in the *B. subtilis* 168 *tatAyCy* and total-*tat* mutant strains was slower and stalled, and this phenotype was complemented by ectopic expression of *tatAyCy*. However, the strains lacking *tatAyCy* did form pellicles after 10 days of prolonged incubation (Figure 1b). This delayed pellicle formation phenotype was not observed for *tatAyCy* mutants of strain 3610, which is probably due to the fact that this strain is much more efficient in pellicle formation than strain 168. Further, the pellicle phenotype of strain 168 related specifically to the TatAyCy translocase, because the absence of *tatAyCy* was not complemented by ectopic expression of the *tatAdCd* genes (data not shown).

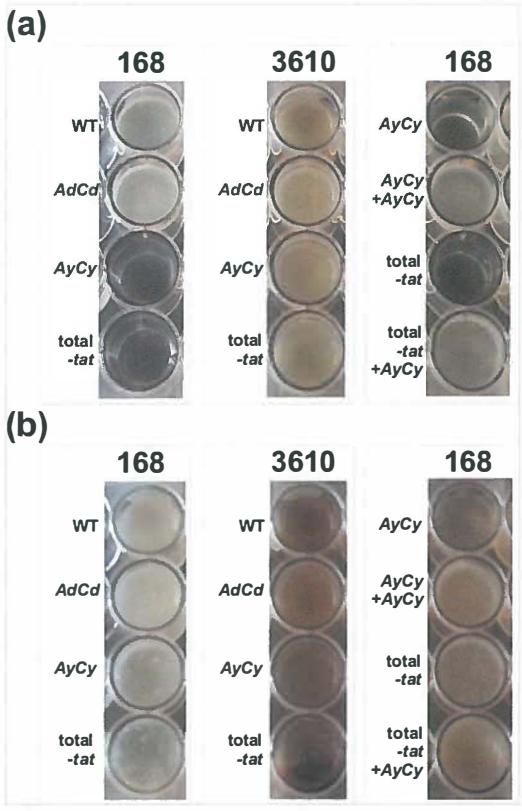

Figure 1. Pellicle biofilm formation by *B. subtilis* 168 and 3610 *tat* mutant strains grown on MSgg media. (a) Pellicle formation after 5 days of incubation. Strains lacking *tatAyCy* (wells labeled *AyCy*) or all *tat* genes (wells labeled total-*tat*) show a delayed pellicle formation, which is complemented by ectopic *tatAyCy* expression (wells labeled +*AyCy*). **(b)** Pellicle formation after 10 days.

Identification of QcrA as a substrate for the TatAyCy translocase

Previous low-stringency RR-signal peptide predictions suggested 69 potential Tat substrates in *B. subtilis* (25). Therefore, in the present studies a hypothesis-driven approach was followed where the effects of tat mutations on these 69 proteins were specifically assessed. Unfortunately, the majority of the potential Tat substrates were not detected by LC-MS/MS, and only 23 could be quantifiably analysed in at least of one the cellular compartments (Table 5). Of these 23 proteins, only YrpE, OpuBC, PelB, LytD, YvhJ, LipA and YvrC showed fold changes (Table 5 and Supplementary Table S1).

Table 5. Hypothesis-driven results of quantitative proteomic analyses of the cytoplasmic, membrane and

	Cytoplasm				Membrane				Extracellular			
	WT	*AdCd*	*AyCy*	total *tat*	WT	*AdCd*	*AyCy*	total *tat*	WT	*AdCd*	*AyCy*	total *tat*
LipA	1.05	1.04	1.10	-1.04	D	D	D	-	D	D	**-1.76**	1.20
YqzC	D	-	-1.02	D	1.04	-1.03	-	1.03	-1.05	1.41	-1.04	-
AspB	1.00	-	-1.06	D	-	-	-	-	-	-	D	D
OppA	-1.03	1.02	-1.02	1.01	-1.04	1.07	D	D	1.14	-1.31	1.00	-1.12
OppB	-	-	-	-	-1.10	1.09	-1.14	D	-	-	-	-
DltD	-	-	-	-	1.22	1.07	1.20	1.02	-	-	-	-
AppB	-	D	-	D	1.10	1.47	1.04	1.01	-	-	-	-
YrpE	D	-	-	-	**-2.00**	Not in ^{15}N	**-1.96**	Not in ^{15}N	1.03	-	**-3.45**	D
YubF	-	-	-	-	1.23	D	D	D	-	-	-	-
MreC	-	-	-	-	-1.05	-1.02	-1.02	-1.05	-	-	-	-
Yvhj	-	-	-	-	D	D	-1.05	1.05	**-1.78**	D	**-2.85**	**-2.17**
YvrC	-	-	-	-	D	D	**-1.88**	D	-	-	-	-
QcrA	-	-	-	-	-	**1.53**	-	-	D	D	Not in ^{15}N	Not in ^{15}N
YdjN	-	-	-	-	1.01	D	0.99	D	-	-	-	-
YrrL	-	-	-	-	1.03	D	D	D	-	-	-	-
LytD	-	-	-	-	-	-	-	-	**-2.77**	**-3.84**	**-2.94**	D
opuBC	-	-	-	-	-	-	-	D	**-1.56**	**-1.66**	D	D
PelB	-	-	-	-	-	-	-	-	**-2.43**	**-1.75**	D	-1.38
WprA	-	-	-	-	-	-	-	-	D	-	D	-1.04
YhcR	-	-	-	-	-	-	-	-	D	-1.31	-1.45	D
YfkN	-	-	-	-	-	-	-	-	D	-1.20	D	D
BglS	-	-	-	-	-	-	-	-	1.03	D	D	D
WapA	-	-	-	-	-	-	D	D	1.17	-	1.03	D
YwbN	Not in ^{14}N	-	Not in ^{14}N	-	-	-	-	-	D	D	-	-

Fold changes represent the differences between the ^{14}N-labeled 168 strain and the four ^{15}N-labeled strains. A fold change of more than 1.5 was considered significant and is indicated in bold. The predicted localization of the protein and its predicted function are indicated. '-': Not Detected, D: Detected, Not in ^{14}N: Not Detected in the ^{14}N-labeled 168 reference strain, Not in ^{15}N: not detected in ^{15}N-labeled strain

Of the predicted Tat substrates identified, one potential substrate named QcrA stood out, because it was identified in the extracellular fraction of the ^{14}N-labeled reference strain, but not in extracellular fractions of the ^{15}N-labeled *tatAyCy* and total-*tat* mutant strains (Table 5). It should be noted here that, due to the requirements and restrictions in the hypothesis-driving MS/MS data analysis approach described above, QcrA was initially filtered out. However, the subsequent hypothesis-driven data assessment suggested that QcrA was secreted in a TatAyCy-dependent manner.

QcrA has been previously characterised as the Rieske iron-sulphur subunit of the cytochrome bc_1 complex (186), and it is predicted by the TOPCONS algorithm (http://topcons.net/) (211) to have a single transmembrane region with an N_{in}-C_{out} topology. In order to examine the dependence of QcrA on the Tat system for membrane translocation, a polyclonal rabbit antibody was generated. As shown by Western blotting, the presence of QcrA-specific bands in whole cell and growth medium fractions was strictly dependent on the *tatAyCy* genes (Figure 2a). Compared to the cell-associated QcrA, the soluble extracellular from of QcrA (labelled QcrA*) had a faster electrophoretic mobility, indicating that the transmembrane region was proteolytically processed. Mutation of the *tatAdCd* genes did not affect the localization of QcrA, and ectopically expressed *tatAdCd* genes were unable to complement the QcrA defect of *tatAyCy* mutant strains (Figure 2a). Interestingly, further fractionation of the cells showed that cell-associated QcrA is specifically localized in the membrane (Figure 2b). No cytoplasmic QcrA was detectable even in *tatAyCy* mutant cells that did not contain membrane-associated QcrA.

The smaller QcrA* fragment observed in growth medium fractions (Figure 2a) corresponded to the predicted protein size without the N-terminal transmembrane region. Since this was reminiscent of signal peptide processing (112), the processing of QcrA was further investigated in strains lacking one or more signal peptidases. Notably, there are five signal peptidases present in *B. subtilis*, denoted as SipS, SipT, SipU, SipV, and SipW (212). These signal peptidases have partially overlapping specificities, are individually not essential, and as much as four of the five signal peptidase can be removed before cells become non-viable (188, 212). As shown in Figure 2c, QcrA* was no longer observed in the growth medium when four of the five signal peptidases were removed (i.e. SipSUVW or SipTUVW). This implies that signal peptidase activity is responsible for release of the QcrA* fragment into the growth medium. Together these findings show that the TatAyCy translocase is needed for stable incorporation of QcrA into the membrane, and that the subsequent release of a smaller derivative form into the growth medium is due to signal peptidase activity.

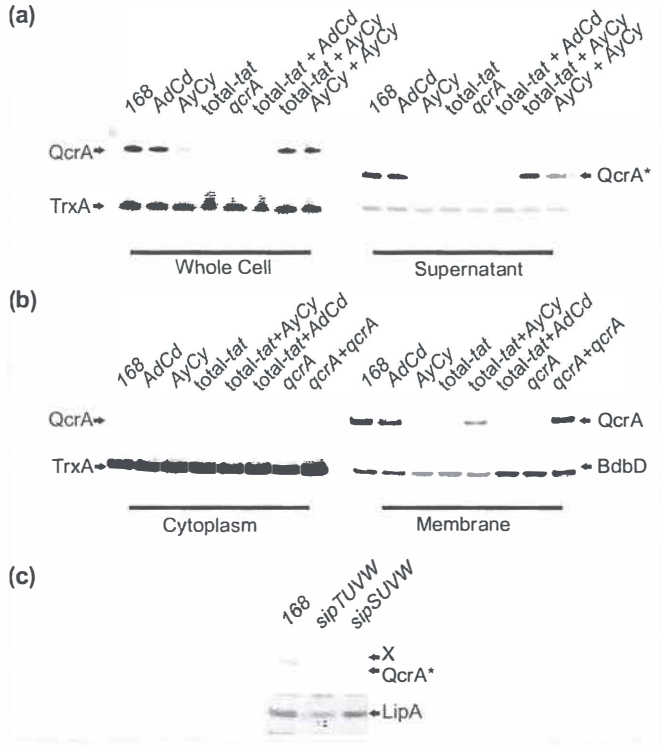

Figure 2. TatAyCy-dependent membrane biogenesis and export of QcrA. The presence of QcrA was assessed by Western blotting analyses with the various *B. subtilis* mutant strains. (a) The presence of QcrA in cellular and extracellular fractions is specifically dependent on TatAyCy. The blot was probed with antibodies specific for QcrA and the cytoplasmic marker protein TrxA. The positions of full-length QcrA, an extracellular processed form of QcrA (labeled QcrA*) and TrxA are marked with arrows. **(b)** Cytoplasmic and membrane fractions the localization of full -length QcrA in the membrane. The blot was probed with antibodies specific for QcrA, the cytoplasmic marker protein TrxA and the membrane protein BdbD (all marked with arrows). 168, parental strain 168; *AdCd*, strain lacking *tatAdCd*; *AyCy*, strain lacking *tatAyCy*; total-*tat*, strain lacking all *tat* genes; *qcrA*, strain lacking the *qcrA* gene; *+qcrA*, strain ectopically expressing *qcrA*; *+AdCd*, strain ectopically expressing *tatAdCd*; *+AyCy*, strain ectopically expressing *tatAyCy*. **(c).** Signal peptidase-dependent extracellular localization of QcrA. The presence of a processed form of QcrA (QcrA*) in growth medium fractions of mutant strains lacking four of the five type I signal peptidases of *B. subtilis* was investigated by Western blotting. QcrA* was not observed in the supernatant *sipSUVW* and *sipTUVW* mutants, where four of the five signal peptidase genes of *B. subtilis* are mutated. The blot was probed with antibodies directed against QcrA, and the extracellular protein LipA. X, unidentified protein that cross-reacts with the antibodies directed against QcrA.

Discussion

The Tat system is able to translocate fully folded and cofactor-containing proteins across biological membranes. Although this pathway is present in all kingdoms of life, relatively little is known as to how the substrate proteins are folded and translocated, or which quality control mechanisms are involved. In the present studies a quantitative proteomic approach was taken to examine the protein content of various *tat* mutant strains, because this would allow us to assess the statistical significance of changes in the amounts of particular proteins over a wide range of fold-factors. This is particularly relevant in those cases where the changes appear to be relatively small. Accordingly, strains were metabolically labelled and LC-MS/MS analyses were performed on subcellular fractions representing the cytoplasm, membrane and extracellular milieu. The metabolic labelling generated unbiased quantitative information allowing for comparisons between the amounts of particular proteins present in the different *B. subtilis tat* mutant strains. This data was then further extrapolated to allow for the identification of novel direct or indirect Tat-related responses.

A large coverage of the predicted *B. subtilis* proteome was detected, where 908, 453 and 447 proteins were identified in the cytoplasmic, membrane and growth medium fractions, respectively. Overall no drastic variations in the amount of proteins were observed in the investigated *tat* mutants. This supports the view that *tat* mutations have no major impact on the composition of the *B. subtilis* proteome (25, 132), and that any phenotypes of these mutants would relate to subtle changes. This was to be expected as few physiological differences between *tat*-mutants and wild-type strains have been observed as long as the growth medium contained sufficient amounts of iron (136). Importantly, slight changes in the amounts of various proteins were indeed detected, and these unveiled a delayed biofilm phenotype thereby highlighting the power of quantitative proteomics approaches.

Very little is known about the pre-translocational processing of Tat substrates in *B. subtilis*. Hence changes in the amounts of cytoplasmic proteins could imply roles in Tat chaperoning, folding and quality control. One such possible interacting partner was identified, namely SufS, which is a scaffold protein involved in iron-sulphur protein assembly (213). SufS was significantly decreased in the total-*tat* mutant lacking all five *tat* genes. A second response was identified through the upregulation of DnaJ in the *tatAdCd* and total-*tat* mutants. DnaJ is a chaperone, which is not only associated with protein folding upon heat shockb (100), but DnaJ has also been implicated in iron-sulphur cluster assembly (99).

The TatAdCd translocase is known to actively translocate its substrates in phosphate-limiting environments and, accordingly, the expression of the *tatAdCd* genes is enhanced upon phosphate depletion (131, 132). However, it has been shown that *tatAd* is constitutively expressed at a low level and hence TatAd may be involved in cellular processes under a wider range of conditions (136). Three oxidative stress proteins (AhpC, AhpF, KatA) showed measurable fold changes in the *tatAdCd* mutant strains. Furthermore, a large portion of the changes observed in the cytoplasmic fraction of these strains relate to the metabolic pathway involved in methionine synthesis and uptake, specifically MetE, YitJ, YoaD, MetK, YxjG, MtnW, YxjH, YoaC (for review, see (214)). Notably, previous studies have shown that the methionine biosynthesis pathway is upregulated during oxidative stress (215). Therefore, the decrease in these proteins in the *tatAdCd* and total-*tat* mutant strains suggests that these strains are either less responsive to oxidative stress, or that they produce lower levels of oxidative compounds, such as H_2O_2.

Of the 27 proteins differentially present in the membrane fractions of *tat* mutants, none could be directly linked to the Tat pathway. Nevertheless, the downregulated YoaB protein forms part of the methionine uptake system (214). Also, the chemotaxis- and motility-related proteins SrfAB and TlpB were observed in different amounts (195, 216).

A number of extracellular proteins with unknown functions were either upregulated (YpjP) or downregulated (YbdN, YitM, YqgA, YvyC) in the total-*tat* mutant strain. One of these, YqgA, was substantially decreased in all *tat* mutant strains. This protein is of an unknown function and the only homologous proteins have been identified in other bacilli.

The Tat system has been previously linked to virulence and biofilm formation in the Gram-negative bacteria *E. coli*, *Pseudomonas aeruginosa, Vibrio cholerae*, *Vibrio alginolyticus* and *Legionella pneumophila* (217-221). In *V. alginolyticus* a *tatC* mutation was associated with a decrease in the extracellular protease Asp, which resulted in a biofilm phenotype (217). To the best of our knowledge, no clear link between biofilm-related proteins and Tat-dependent translocation had as yet been identified in Gram-positive bacteria. The subtle Tat-related phenotype in pellicle formation observed in the present study was identified through the effects of the *tatAyCy* mutation on the biofilm-associated proteins TasA, YoaW, Epr, LytF, LytA/B. However, the effects of the TatAyCy translocase on these proteins cannot be easily explained with what is known regarding the biofilm pathway. Clearly, the biofilm formation pathway is part of much larger complex gene regulatory and protein interaction networks with a substantial number of unknown ele-

ments (222). One of these could be the TatAyCy translocase, which was recently shown to be expressed highly under biofilm forming conditions (131). Therefore, the delayed pellicle forming phenotype observed for the *tatAyCy* mutant strains could be related to an as yet unidentified Tat substrate, or to as yet undefined indirect interactions.

The hypothesis-driving approach used in this study was shown to be a powerful tool to identify novel Tat-associated responses. Nevertheless, not all known Tat-related proteins were identified, which reflects the fact that not all proteins are readily detectable or quantifiable by proteomics. This was underscored by the lack of detection of the expressed Tat substrate YwbN. However, by approaching the data in both hypothesis-driving and -driven manners, this shortcoming was partially resolved and by looking specifically for potential substrates, the *B. subtilis* QcrA protein was identified and confirmed to be a Tat substrate. QcrA represents the Rieske iron-sulphur subunit of the cytochrome bc_1 complex (186), and its twin-arginine signal peptide was previously shown to direct efficient protein export in *Streptomyces* (223). Furthermore, QcrA was shown to be Tat-dependently translocated into the chloroplast thylakoid membrane (114), and the inner membrane of some Gram-negative bacteria, such as *Legionella pneumophilia* (115) and *Paracoccus denitrificans* (116). Notably, the cytochrome bc_1 complex is non-essential in *Bacillus* (186), which explains why the absence of QcrA upon elimination of the TatAyCy translocase had no measurable impact on cell physiology under the tested conditions. In this study we also showed that the proteolytic processing of QcrA and subsequent release of a smaller form into the growth medium was associated with the presence of several signal peptidases of *B. subtilis*. However, it remains unclear whether this processing of QcrA is physiologically relevant.

In conclusion, the present studies have generated a global overview of the Tat proteome in *B. subtilis*. This not only generated a rich minable data set, but also led to the identification of QcrA as a novel Tat substrate in *Bacillus* and the involvement of the Tat pathway, directly or indirectly, in biofilm-associated pellicle formation. Altogether, by following a quantitative proteomics approach, twin-arginine translocation pathway-dependent phenotypes were unveiled.

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Supporting Information is detailed on Journal of Proteome website: http://pubs.acs.org/journal/jprobs

Supplementary Table S1 –Fold changes in protein levels associated with the deletion of *tatAdCd*, *tatAyCy*, or all *tat* genes

Supplementary Table S2 – Fold changes in protein levels associated with induced expression of YwbN

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Chapter 3

A Tat ménage *à* trois - the role of TatAc in *Bacillus subtilis* twin-arginine translocation

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Abstract

The twin-arginine translocation system (Tat) is a protein transport system that moves fully folded and co-factor-containing proteins across membranes of bacteria, archaea and thylakoids. In the Gram-positive *Bacillus subtilis* bacterium, the minimal Tat pathway is composed of two subunits TatA and TatC. TatC is a large transmembrane protein that associates with Tat substrates before recruiting TatA. TatA is a protein with a single transmembrane domain, and a number of TatA subunits combine to from a pore for protein translocation. There are two TatC subunits encoded by the *B. subtilis* genome (TatCd and TatCy) and three TatA subunits (TatAd, TatAy and TatAc). These subunits combine to form two parallel TatA-TatC translocases, TatAy-TatCy and TatAd-TatCd. Although it is consistently expressed, the purpose and role of the third TatA component, TatAc, has remained ambiguous. In this study, we have examined a growth phenotype directly associated with the translocation of the TatAy-TatCy-dependent substrate EfeB (YwbN) in various Tat-deficient genetic backgrounds. We examined the ability of different TatAy mutant subunits to complement for the absence of wild-type TatAy. In doing so, it was observed that in instances where specific amino acid substitutions were made in the amphipathic helix region of TatAy a definite growth phenotype was observed only in genetic backgrounds lacking TatAc. Altogether, our findings imply that TatAy and TatAc interact and that, although not essential *per se*, this TatAy-TatAc interaction effectively supports the translocation of the Tat substrate EfeB when TatAy function is compromised. This suggests that the third TatA-like protein in *B. subtilis* might represent an intermediate evolutionary step in TatA-TatB specialization.

Introduction

Twin-arginine translocation systems (Tat) transport fully folded and co-factor attached proteins across the membranes of thylakoids, archaea, Gram-positive or Gram-negative bacteria (9-11). The proteins targeted to the Tat system contain a specific N-terminal signal peptide with a characteristic twin-arginine (RR-)motif (11, 35). Phylogenetic comparisons of Tat systems showed that the basic Tat system is most often composed of a TatC and two sequence-diverged TatA-like proteins (48). The two TatA-like components are a result of a direct gene duplication where the second copy is found elsewhere on the genome and has, in some cases, diverged drastically (36, 48). This diverged evolution is illustrated well in the Tat system of the Gram-

negative bacterium *Escherichia coli*. Here the TatA-like proteins have evolved to different functions, namely substrate docking (TatB) or pore formation (TatA) (43, 44, 57, 58, 73, 74). On the other hand, at least two of the TatA components of the Gram-positive bacterium *Bacillus subtilis* can perform both functions (23, 24).

TatC is a relatively large integral membrane protein with six transmembrane domains (42, 224). In contrast, TatA-like proteins are smaller with a single transmembrane-spanning domain followed by a hinge region, an amphipathic helix and a densely charged C-terminal tail (8, 60, 79-81). In the current consensus model of translocation, the process is initiated when a substrate with the correct RR-signal peptide interacts with the docking complex composed of TatC and a TatA-like protein. In *E. coli* this TatA-like protein has become specialized and is known as TatB (48, 71, 82). The docking complex interacts specifically with the signal peptide (16, 40-42, 225), has been implicated in proofreading (11, 43), and inserts the substrate into the membrane (44). This docking complex then recruits the pore-forming TatA components (16, 179) and the proton-motive force is used as an energy source for translocation (40, 226, 227).

B. subtilis is a Gram-positive model organism and a biotechnologically relevant bacterium associated with a high capacity to secrete proteins (12). The core Tat pathway in *B. subtilis* has been defined as a single TatA and TatC pair (72). However, genes for two TatC (TatCd and TatCy) and three TatA components (TatAd, TatAy and TatAc) have been identified on the *B. subtilis* genome. These Tat components can combine to form two parallel TatA-TatC translocases, TatAy-TatCy (TatAyCy) and TatAd-TatCd (TatAdCd). Each pathway works independently of the other, translocating its own substrates (72, 132). The *tatAyCy* and *tatAdCd* pairs are expressed operonically. Large-scale expression studies (130, 131) have shown that *tatAdCd* is expressed only under conditions of low phosphate, corresponding to the expression of its one known substrate PhoD, a phosphodiesterase (132, 133). In contrast, *tatAyCy* is expressed consistently over many tested conditions and has a broader substrate specificity including the Dyp-type peroxidase EfeB, the Rieske iron-sulphur protein QcrA and the alkaline phosphatase YkuE (20, 72, 129).

The role of the third *B. subtilis* TatA component, TatAc, has remained more ambiguous. Although it is consistently expressed (131), TatAc mutant strains have not shown any phenotype (14, 25, 72). Nor could TatAc compensate for the absence of TatAy or TatAd when translocating EfeB or PhoD respectively (134). Nevertheless, TatAc has shown functionality in *E. coli*, as active TatAc-TatCd or TatAc-TatCy complexes translocated the *E. coli* Tat

substrates AmiA, AmiC and TorA (61). Also, TatAc was able to compensate for the absence of *E. coli* TatA and TatB (59). Protein-protein interaction studies with Yeast two-hybrid (Y2H) techniques have shown that TatAc not only interacts directly with itself, with TatAd and TatAy, but also with the HemAT protein recently implicated in PhoD secretion (98). Combined, these recent studies have implicated TatAc in protein translocation, but a precise role for TatAc in *B. subtilis* Tat-dependent translocation had yet to be defined.

Under nutrient-rich conditions no major phenotypes associated with the Tat system have been observed (72). Notably, under conditions of limited iron or NaCl availability clear growth phenotypes are observed (132, 136). These phenotypes are directly associated with the Tat-dependent substrate EfeB and its role in ferric iron scavenging (72, 136). For the purpose of the work presented here, advantage has been taken of the drastic lysis phenotype of *tatAyCy* or *efeB* mutant cells observed under low salt growth conditions (136). In this study we investigated the export of EfeB by TatAy and TatCy, where single amino acid residues in TatCy or TatAy were systematically mutated. From the growth phenotypes observed, we were able to assess the importance of specific amino acid residues with regards to the active translocation of EfeB. Importantly, we were able to grade the efficiency of this translocation by the severity in the growth phenotype. In doing so, a role for TatAc in the active translocation of EfeB was uncovered.

Materials and Methods

Bacterial strains, plasmids, media and basic growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Lysogeny Broth (LB) was composed of 1% tryptone and 0.5% yeast extract with or without 1% NaCl. The amount of NaCl used is specified for each experiment. Bacteria were grown in LB broth at 37 °C under vigorous shaking, or on LB agar plates incubated at 37 °C. When appropriate, the cultures were supplemented with 100 µM $FeCl_3$ or antibiotics: *E. coli* cultures with 100 µg/ml ampicillin (Ap) and *B. subtilis* cultures with 2 µg/ml erythromycin (Em), 5 µg/ml chloramphenicol (Cm), 10 µg/ml tetracycline (Tc), 100 µg/ml spectinomycin (Sp), or 20 µg/ml kanamycin (Km). *B. subtilis* cells were grown to competence in Paris Medium (PM) and transformations were performed as previously described (168).

Table 1. Strains and plasmids used in this study

Strains	Relevant properties	Ref
B. subtilis 168	*trpC2*	(185)
B. subtilis 168 tatAc$_1$	*trpC2; tatAc::Em; Em^r*	(25)
B. subtilis 168 tatAdCd	*trpC2, tatAd-tatCd::Km; Km^r*	(25)
B. subtilis 168 tatAyCy	*trpC2, tatAy-tatCy::Sp; Sp^r*	(72)
B. subtilis 168 tatAdCd - tatAyCy	*trpC2; tatAd-tatCd::Km, tatAy-tatCy::Sp; Sp^r, Km^r*	This study
B. subtilis 168 tatAc$_1$-tatAyCy	*trpC2; tatAc::Em, tatAy-tatCy::Sp; Em^r; Sp^r*	(25)
B. subtilis 168 tatCd-tatCy	*trpC2; tatCd::Km; tatCy::Sp; Km^r; Sp^r*	(132)
B. subtilis 168 total-tat$_2$	*trpC2, tatAd-tatCd::Km, tatAy-tatCy::Sp; tatAc::Em^r; Sp^r, Km^r, Em^r*	(72)
Plasmids		
pHB-*AyCy*	pHB201-derivative containing the *tatAy-tatCy operon; Em^r; Cm^r*	(136)
pGDL48	pGDL41 derivative lacking the *sipS gene and containing an mcs; Km^r*	(134)
pC-Ac	pGDL48 derivative containing the *tatAc gene; Ap^r; Km^r*	(228)
pHB-TatC^{aa}	pHB201-derivatives carrying *tatCy genes that specify mutant TatCy proteins with specific single amino acid substitutions or C-terminal deletions; Em^r; Cm^r*	(68)
pHB-TatA^{aa}TatCy^{WT}	pHB201-derivatives carrying a wild-type copy of the *tatCy gene plus tatAy genes that specify mutant TatAy proteins with specific single amino acid substitutions; Em^r; Cm^r*	(83)

Growth assay associated with iron scavenger deficiency

Phenotypes associated with the absence of active EfeB were monitored by optical density readings at 600 nm in microtiter plate readers (Biotek Synergy 2) using an adapted methodology described previously (131). Strains were grown overnight in LB medium with 1% NaCl and appropriate antibiotics. Cultures were then diluted 50-fold into fresh LB with 1% NaCl in 96 well microtiter plates and grown till mid-exponential phase (approximately 3 h). Cultures were then diluted 50-fold in LB without NaCl but supplemented with freshly prepared 100 µM $FeCl_3$ and grown till mid-exponential phase. A final 50-fold dilution was made into LB without NaCl and without iron sup-

plementation, and growth was monitored for 14 h. Each strain was grown in triplicate within each experiment and each experiment was repeated five to ten times.

Results

Growth defects of *B. subtilis* associated with the absence of EfeB and/or TatAyCy have previously been reported for conditions of iron limitation or low salt (136). In particular, it was shown that strains lacking TatAyCy or EfeB displayed a strong lysis phenotype in LB medium without NaCl. This phenotype was fully complemented by reintroducing TatAyCy or EfeB, respectively, or by the addition of 100 µM ferric iron ((136)and Figure 1a). For the purpose of dissecting the impact of different *tat* mutations on growth in the absence of salt, in this study all strains were pre-cultured in LB without NaCl supplemented with 100 µM $FeCl_3$. This allowed the strains to pre-adapt to a low salt environment so that any growth effects observed would correlate to the deficiency in the export of active EfeB via TatAyCy. Importantly, this preculturing allowed us to observe and categorize new phenotypes associated with site-specific TatCy or TatAy mutations as detailed in the following sections.

Mutations in the Tat system cause a deficiency in active EfeB secretion resulting in graded growth phenotypes

The secretion of EfeB by strains with site-specifically mutated TatCy proteins has been previously analysed by Western blotting and the results are summarized in Table 2 (68). Here a total of 13 mutant TatCy proteins were generated, of which 11 contain amino acid substitutions located on the first or second intracellular loop (referred to as $TatCy^{aa}$), and two have a shortened C-terminus lacking either 5 ($TatCy^{c\text{-}5}$) or 8 ($TatCy^{c\text{-}8}$) residues (68). The strains used to test the functionality of these TatCy mutant proteins had both *tatC* genes deleted from the chromosome and expressed individual TatCy mutant proteins off a plasmid. In the present study, the growth of these strains was monitored by optical density readings in LB media without NaCl.

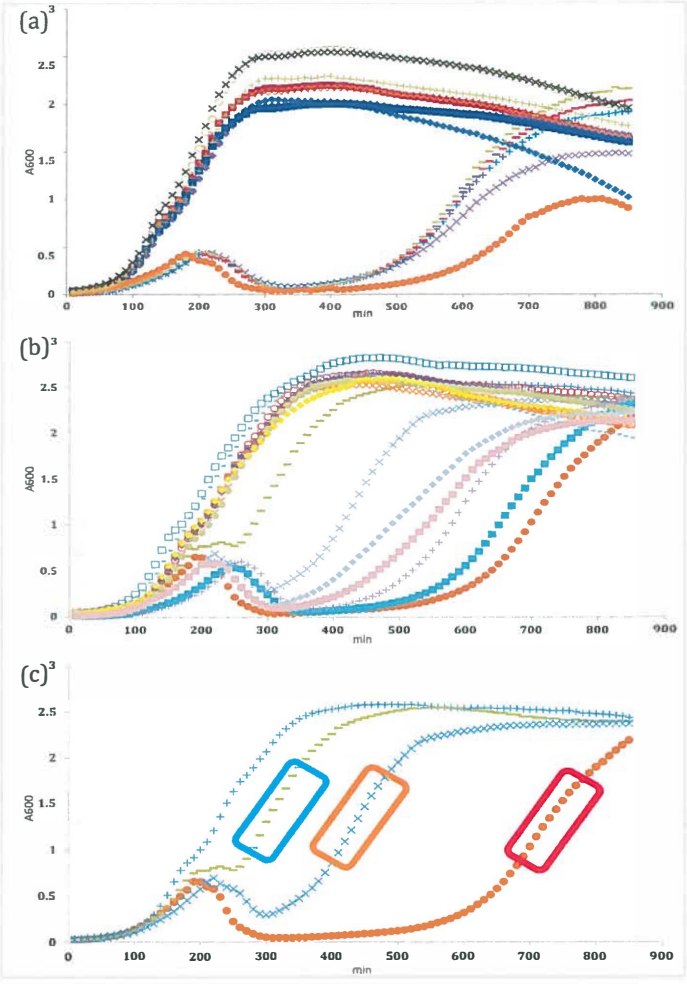

Figure 1. Growth of strains expressing mutant TatCy proteins in LB without NaCl. (a) Growth of *tat* mutant strains was measured as the absorbance at 600nm (A_{600}). The investigated strains are represented as followed: *B. subtilis* 168 (◆), *tatAc* (■), *tatAdCd* (○), *tatAyCy* (×), *tatAdCd-tatAyCy* (+), *tatAc-tatAyCy* (–), *total-tat* (–), *tatCd-tatCy*(•), *tatAyCy* + pHB-AyCy (◆), *tatAdCd-tatAyCy* + pHB-AyCy (■), *tatAc-tatAyCy* + pHB-AyCy (□), *total-tat* + pHB-AyCy (□), *tatCd-tatCy* + pHB-AyCy (+). **(b)** Growth of strains expressing site-specifically mutated TatCy proteins grown in LB without NaCl. Strains are represented as: *B. subtilis* 168 (□), *tatCd-tatCy* mutant background (•), *tatCd-tatCy* mutant background with pHB-*tatCy*$^{\text{wild-type}}$ (+), pHB-*tatCy*$^{\text{L11A}}$ (○),pHB-*tatCy*$^{\text{H14A}}$ (–), pHB-*tatCy*$^{\text{I15A}}$ (◆), pHB-*tatCy*$^{\text{L18A}}$ (■), pHB-*tatCy*$^{\text{R19A}}$ (□), pHB-*tatCy*$^{\text{K20A}}$ (□), pHB-*tatCy*$^{\text{P96A}}$ (×), pHB-*tatCy*$^{\text{G97A}}$ (•), pHB-*tatCy*$^{\text{L98A}}$ (+), pHB-*tatCy*$^{\text{E102A}}$ (–), pHB-*tatCy*$^{\text{S108A}}$ (◆), pHB-*tatCy*$^{\text{C-5}}$ (◆), or pHB-*tatCy*$^{\text{C-8}}$ (■). **(c)** Simplified graphical representation of the graded severity of growth phenotypes. Blue, mild phenotype; orange, severe phenotype; red, very severe phenotype.

Table 2. Summary of graded growth phenotypes observed for strains producing particular TatCy mutant proteins.

	EfeB-myc secretion (68)	Growth phenotype in tatCd-tatCy
168	+	WT
ΔCdCy	-	Very severe
Δ+Cy-WT	+	WT
Δ+Cy-L11A	+	WT
Δ+Cy-H14A	-	Mild
Δ+Cy-I15A	+	WT
Δ+Cy-L18A	-	Very severe
Δ+Cy-R19A	+	WT
Δ+Cy-K20A	-	Severe
Δ+Cy-P96A	+	WT
Δ+Cy-G97A	+	WT
Δ+Cy-L98A	-	Very severe
Δ+Cy-E102A	+	WT
Δ+Cy-S108A	+	WT
Δ+Cy-C5	-	Very severe
Δ+Cy-C8	-	Very severe

The specific amino acid mutations within TatCy are indicated. Secretion of EfeB-myc was previously monitored by Western blotting using an anti-myc antibody: +, EfeB-myc detected in the growth medium; -, no EfeB-myc detected in the growth medium. Growth phenotypes are summarized as follows: WT: growth equivalent to wild-type strain. WT*: in some assays a very mild phenotype was observed, however this was not consistently detectable in every assay. Mild: a phenotype was observed as a small dip at 200 minutes with immediate recovery. Severe: a phenotype that showed a strong drop in optical density and a recovery between 200 and 300 minutes into the assay. Very severe: a phenotype where growth did not recover well over 300 minutes into the assay.

A mild growth phenotype was observed for the strain expressing TatCy^{H14A}. In contrast, the strain expressing TatCy^{K20A} showed a severe phenotype, and strains expressing TatCy proteins with the L18A, L98A, C-5 or C-8 mutations showed very severe growth phenotypes (Figure 1b). Despite their growth defects, all strains were able to recover within 14 hours, implying an adaptive response. This adaptation was not sustained because, upon re-inoculation of the strains after recovery, they showed similar growth phenotypes as before (Supplementary Figure 1). This is consistent with the previously documented EfeB export deficiency of the different TatCy mutant proteins (68).

Importantly, the growth of each strain was very consistent between repeated experiments and phenotypes could be clearly categorized. Depending on the strains' ability to recover, phenotypic grades were observed (Figure 1c); a mild phenotype where the strain was able to recover quite quickly, a severe growth phenotype where the optical density dropped drastically and recovered between 200 and 300 minutes, and a very severe phenotype where strains were only able to recover after 300 minutes or longer. This grading of phenotype severity is represented in Table 2. Notably, the growth phenotype observed for each strain matched the EfeB export defect of the respective TatCy mutant protein as previously assessed

A role for TatAc in EfeB translocation is implied in strains with deficiencies in multiple TatA components

To determine the effects of TatAy mutations on growth in LB without salt, we employed twelve previously generated TatAy mutant proteins with site-specific amino acid substitutions (TatAy^{aa}). An operon composed of mutated *tatAy*^{aa} and wild-type *tatCy* was reintroduced into *tatAy* mutant strains via an expression plasmid. These amino acid substitutions in TatAy span the whole protein; two amino acid substitutions were generated in the N-terminal region (P2A and P2D), four amino acid substitutions were located in the hinge region (F19A, G20A, P21A, K23A), and six amino acid substitutions were in the amphipathic helix region (L24A, G28A, A31G, G32A, F38A, N40A) (83).

Growth effects of the mutant TatAy^{aa} proteins were observed in various *tat* mutant backgrounds and differences in phenotypes were observed depending on the genetic backgrounds, as summarized in Table 3. These mutant backgrounds included strains where individual pathways were removed (i.e. *tatAyCy* or *tatAdCd*), both pathways were removed (*tatAdCd-tatAyCy*), the TatAyCy pathway was removed in combination with TatAc (*tatAc-tatAyCy*), or all Tat components were removed (total-*tat*). As described for the TatCy mutant proteins, phenotypes of graded severity were observed (Figure 3b-e). Further, upon re-inoculation of the strains after recovery, they showed similar growth phenotypes as before (Supplementary Figure 1). In *tatAyCy* or *tatAdCd-tatAyCy* genetic backgrounds the growth phenotypes observed for particular TatAy mutant proteins were very similar (Figure 2a and 2b). Specifically, strains producing wild-type TatCy and TatAy^{P21A}, TatAy^{L24A} or TatAy^{P2A} severe growth phenotypes, and the strains producing TatAy^{G20A} showed a very severe growth phenotype. On the other hand, the majority of strains lacking the chromosomal *tatAyCy* or *tatAdCd-tatAyCy* genes, but expressing the P2D, A31G or G32A mutant proteins TatAy plus wild-type TatCy showed extremely mild growth phenotypes if any.

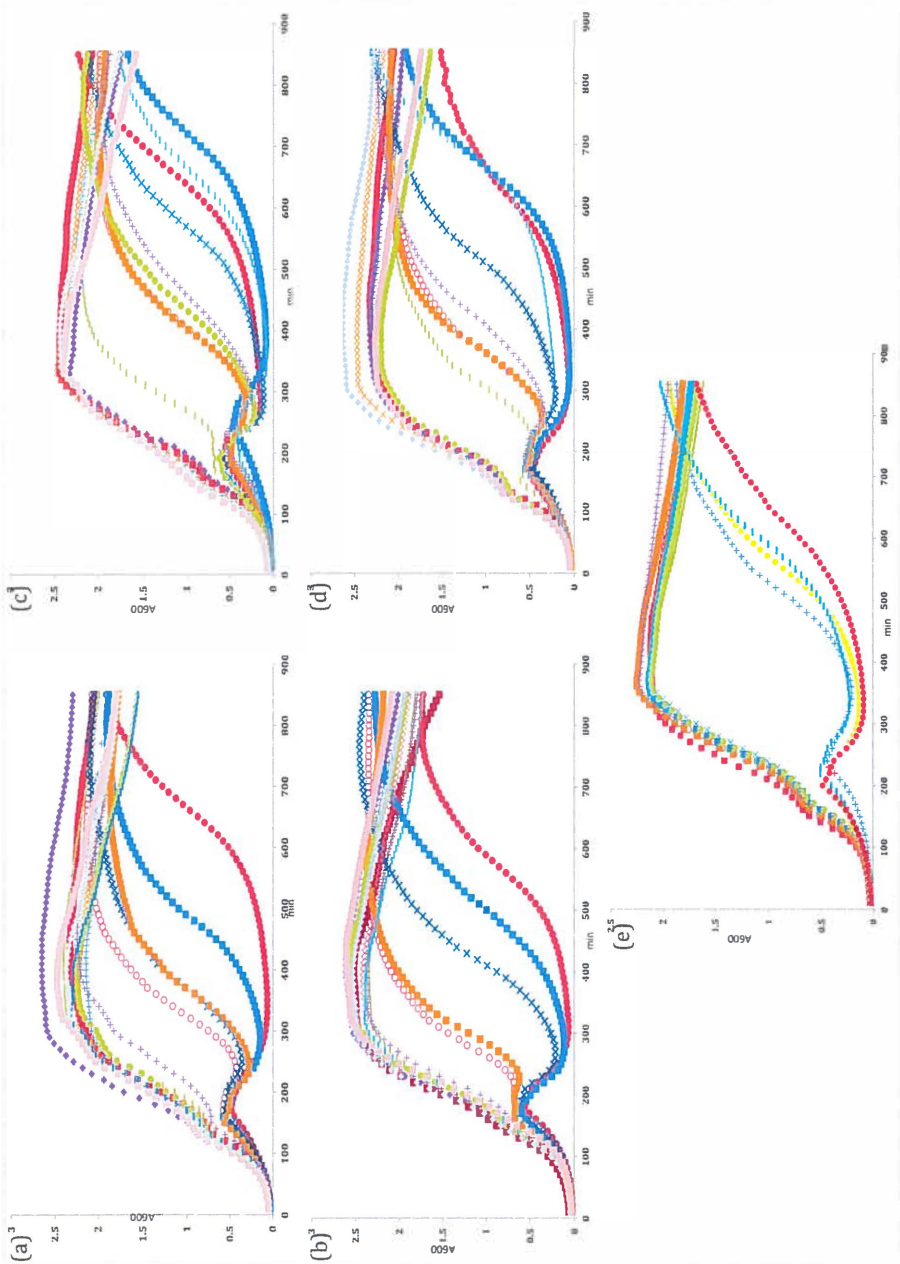
(a)
(b)
(c)
(d)
(e)
A600
min

Figure 2 (adjacent page) Growth of strains producing mutant TatAy proteins in LB without NaCl. The different panels represent the results obtained with strains lacking particular chromosomal *tat* genes that were complemented with site-specifically mutated TatAy proteins plus a wild-type TatCy protein. The chromosomal *tat* mutations included: **(a)** *tatAyCy*, **(b)** *tatAdCd-tatAyCy* **(c)** *tatAc-tatAyCy* **(d)** total-*tat*. Strains carrying plasmids for the expression of particular TatAy mutant proteins are represented as follows: *B. subtilis* 168 (■), mutant background without any plasmid (•), mutant background with pHB-Ay$^{wild\text{-}type}$Cy$^{wild\text{-}type}$ (+), pHB-Ay^{P2A}Cy$^{wild\text{-}type}$ (○), pHB-Ay^{P2D}Cy$^{wild\text{-}type}$ (–), pHB-Ay^{F19A}Cy$^{wild\text{-}type}$ (◆), pHB-Ay^{G20A}Cy$^{wild\text{-}type}$ (■), pHB-Ay^{P21A}Cy$^{wild\text{-}type}$ (□), pHB-Ay^{K23A}Cy$^{wild\text{-}type}$ (□), pHB-Ay^{L24A}Cy$^{wild\text{-}type}$ (■), pHB-Ay^{G28A}Cy$^{wild\text{-}type}$ (•), pHB-Ay^{A31G}Cy$^{wild\text{-}type}$ (+), pHB-Ay^{G32A}Cy$^{wild\text{-}type}$ (–), pHB-Ay^{F38A}Cy$^{wild\text{-}type}$ (◆), pHB-Ay^{N40A}Cy$^{wild\text{-}type}$ (■). Panel **(e)** shows the growth curves of strains with the *tatAc-tatAyCy* genetic background complemented with pHB-Ay^{aa}Cy$^{wild\text{-}type}$ and pC-Ac (carrying *tatAc*). *B. subtilis* 168 (■), *tatAc-tatAyCy* without any plasmids (•), *tatAc-tatAyCy* with the empty vector pGDL48 (•), *tatAc-tatAyCy* with pC-Ac only (+), *tatAc-tatAyCy* with pC-Ac and pHB-Ay^{P2A}Cy$^{wild\text{-}type}$ (○)/ pHB-Ay^{P2D}Cy$^{wild\text{-}type}$ (–)/pHB-Ay^{G20A}Cy$^{wild\text{-}type}$ (■)/pHB-Ay^{P21A}Cy$^{wild\text{-}type}$ (□)/pHB-Ay^{L24A}Cy$^{wild\text{-}type}$ (■)/ pHB-Ay^{G28A}Cy$^{wild\text{-}type}$ (•)/ pHB-Ay^{A31G}Cy$^{wild\text{-}type}$ (+)/ pHB-Ay^{G32A}Cy$^{wild\text{-}type}$ (–)

Intriguingly, the growth of *tatAc-tatAyCy* or total-*tat* strains expressing particular mutant TatAy proteins plus wild-type TatCy showed a number of important differences when compared to the *tatAyCy* or *tatAdCd-tatAyCy* strains (Figure 2c and 2d). In both backgrounds where *tatAyCy* and *tatAc* were absent, the growth defect caused by the A31G mutation in TatAy was graded as severe, and the growth defects caused by the G32A or P21A mutations as very severe (Figure 2, Table 3). This implied that the malfunction of these TatAy mutant proteins was exacerbated in the absence of TatAc. To further investigate the possible complementary role of TatAc, a plasmid expressing TatAc was reintroduced into the *tatAc-tatAyCy* strains. Growth assays were performed again, but in this case *tatAc-tatAyCy* was complemented with two plasmids; one expressing *tatAc* (pC-Ac) and the other the modified *tatAyCy* operon (pHB-TatAy^{aa}Cy$^{wild\text{-}type}$). The results of this growth assay are shown in Figure 2e and summarized in Table 3. Notably, the reintroduction of TatAc resulted in growth equal to that of the wild-type strain in all cases except for the strain producing TatAy^{G32A}. This shows that TatAc is able to compensate for the functional defects of all TatAy mutant proteins except the defect of TatAy^{G32A}.

Table 3. Summary of graded growth phenotypes observed for strains producing particular TatAy mutant proteins

	EfeB-myc (83)	Growth phenotype in tatAyCy	Growth phenotype in tatAdCd -tatAyCy	Growth phenotype in tatAc-tatAyCy	Growth phenotype in total-tat	Growth phenotype in tatAc-tatAyCy + pC-Ac
168	++	WT	WT	WT	WT	WT
Δ	-	Very severe	Very severe	Very severe	Very severe	Very severe
Δ+ Ay-WT	++	WT	WT	WT	WT	
Δ+ Ay-P2A	-	Severe	Severe	Severe	Severe	WT
Δ+ Ay-P2D	-	WT*	WT*	Mild	Mild	WT
Δ+ Ay-F19A	++	WT	WT	WT	WT	
Δ+ Ay-G20A	-	Very severe	Very severe	Very severe	Very severe	WT
Δ+ Ay-P21A	-	Severe	Severe	Very severe	Very severe	WT
Δ+ Ay-K23A	+	WT	WT	WT	WT	
Δ+ Ay-L24A	-	Severe	Severe	Severe	Severe	WT
Δ+ Ay-G28A	-	WT	WT	WT	WT	WT
Δ+ Ay-A31G	+	WT*	WT*	Severe	Severe	WT
Δ+ Ay-G32A	+	WT	WT*	Very severe	Very severe	Very severe
Δ+ Ay-F38A	++	WT	WT	WT	WT	
Δ+ Ay-N40A	++	WT	WT	WT	WT	

The specific amino acid mutations within TatAy are indicated. Secretion of EfeB-myc was previously monitored by Western blotting using an anti-myc antibody: ++, wild-type levels of EfeB-myc detected in the growth medium; +, low levels of EfeB-myc detected in the growth medium; -, no EfeB-myc detected in the growth medium. Growth phenotypes for strains lacking different chromosomal *tat* genes and expressing particular plasmid-borne *tat* genes are summarized as follows: WT: growth equivalent to wild-type strain. WT*: in some assays a very mild phenotype was observed, however this was not consistent in every assay. Mild: a phenotype was observed as a small dip at 200 minutes with immediate recovery. Severe: a phenotype that showed a strong drop in optical density and a recovery between 200 and 300 minutes into the assay. Very severe: a phenotype where growth did not recover well over 300 minutes into the assay.

Discussion

In this study we examined the ability of mutated TatAy and TatCy proteins to complement for wild-type TatAy or TatCy in various Tat-deficient genetic backgrounds, illustrated in Figure 3. This unveiled a clear role for TatAc in protein translocation in *B. subtilis*. Specifically, we investigated the phenotypic variation of strains with amino acid substitutions in TatAy or TatCy grown LB without NaCl, where the absence of TatAyCy or EfeB has been shown to result in a clear growth phenotype. Notably, the observed growth phenotypes were directly related to the amounts of active EfeB translocated by the TatAyCy system. These growth phenotypes were graded as mild, severe or very severe. A summary of the results is visualized in Tables 3 and 4. Phenotypes associated with mutated amino acid residues suggest altered interactions between the Tat components themselves, or between the Tat components and their EfeB substrate. The implication that TatAc is involved in translocation adds an extra layer of complexity to the Tat system, because the amino acid substitutions resulting in phenotypes are potentially involved in interactions between TatAy-TatAc or TatAy-TatAc-TatCy and the substrate. In this respect it is noteworthy that Yeast-two-hybrid analyses showed clear evidence for direct interactions between TatAy and TatAc (98).

The graded phenotypes observed imply that the quality of translocation varies between strains and suggests different efficiencies in EfeB translocation and processing. All strains were able to recover from the growth defects eventually and this recovery was not retained after further culturing. Hence, recovery likely relates to an adaptive response regarding iron uptake, or iron metabolism rather than the Tat-system directly (83, 125).

For strains with amino acid substitutions in either TatAy or TatCy resulting in very severe phenotypes, it was suggestive that these amino acid mutants resulted in a complete blockage of active EfeB translocation. This is supported by the severe phenotypes observed in the 'bare' mutant background strains without complementing plasmids. The strains where the phenotype was graded as severe suggested that the mutated Tat component resulted in an inefficient translocation, but not complete blockage. These strains adapted to the environment faster than those with the very severe phenotype. This could correspond to a change in the quality or quantity of active translocated EfeB, potentially due to a severe delay in proofreading, translocation or release of EfeB, which would imply that these amino acid mutations could be central to processing activities, but not essential. The milder phenotypes may suggest that the amino acids mutated play important roles in translocation, but that this is easily compensated for. This assay therefore gave a direct representation of the translocation process in *B. sub-*

tilis and allowed for novel insights into the Tat translocation system of *B. subtilis*.

The TatCy amino acid mutations used in this study correspond to the regions shown to be important in *E. coli* TatC, namely the N-terminal region (42, 62, 65, 229), the first cytoplasmic loop (63-66, 68), and C-terminal tail (68). The phenotypes observed (mild, severe or very severe) corresponded well to the Western blot profile generated in a *B. subtilis* assay where the secretion of EfeB-myc was analysed after xylose-induced expression (68). Therefore, the results relating to TatCy presented here provide further insights regarding the relative importance of amino acid residues in TatCy.

TatAy is a smaller protein than TatCy, but it contains a number of defined structural regions. TatAy is composed of a short N-terminal domain which sticks outward from the membrane, a single transmembrane domain, a short flexible hinge region and an amphipathic helix that is thought to lie flat against the membrane (79-81). The amphipathic helix region leads onto a second flexible end region containing a large number of densely charged residues (60). These structural regions are important as they all have specific roles regarding translocation and specific residues have been shown to be vital for this process (23, 24, 75, 78, 82-85). Specific TatA amino acid mutants have been investigated in *E. coli* TatA (82, 86), and in *B. subtilis* TatAy (83) and TatAd (23). These studies have shown intolerance to mutations particularly in the hinge and amphipathic helix regions of TatA proteins (82, 83, 86). Studies investigating xylose-induced EfeB-myc, in a genetic background where only TatAyCy was absent, showed by Western blotting that a number of residues are specifically important for EfeB translocation (83). The results in these studies corresponded to the graded growth phenotypes presented here, with a few exceptions relating specifically to the hinge and amphipathic region. In the TatAyCy-deficient background, mutations at P2D, G28A, A32G and G32A showed no or very mild growth defects. In the case of these amino acid mutants in *B. subtilis* (83) and the corresponding mutations in *E. coli* TatA, results had suggested more severe phenotypes (82, 86). This led us to believe that other TatA components in *B. subtilis* could be involved with TatAyCy with regards to active EfeB translocation.

Intriguingly, when specific TatAy mutations were investigated in different *tat* genetic backgrounds, some phenotypes were exacerbated in strains specifically devoid of TatAc. In the *tatAc-TatAyCy* and total-*tat* strains, TatAy mutations at P2D, A31G or G32A showed distingct phenotypes whereas this was not the case in strains containing *tatAc*. The phenotype associated with P21A was graded from severe to very severe. In the case of the residues in the amphipathic helix that showed variation, namely A31G and G32A,

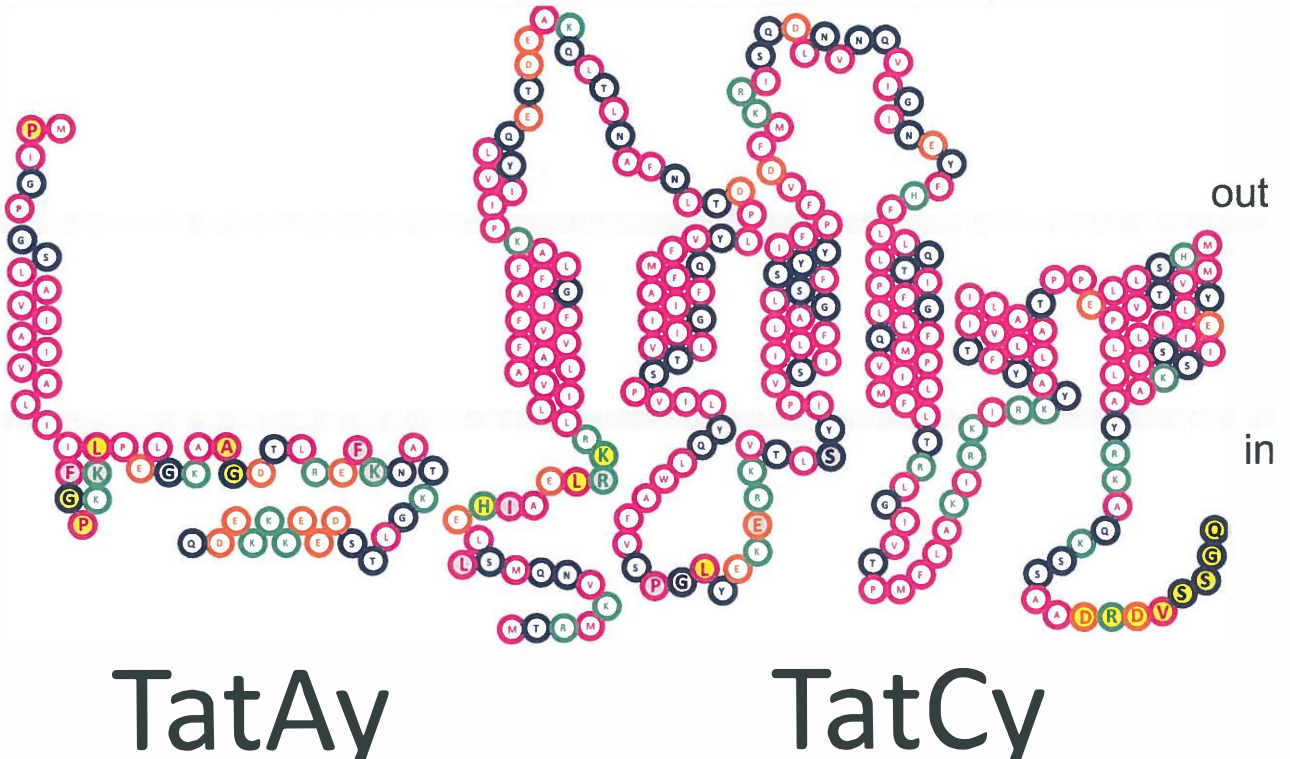

Figure 3. Graphical representation of TatAy and TatCy and the amino acid substitutions investigated in this study. Mutated amino acid residues are shaded in grey and those mutated residues that resulted in a graded growth phenotype are shaded yellow. Further, hydrophobic non-polar residues are coloured pink, polar basic residues green, polar acid residues are coloured orange, uncharged polar residues are in blue. In, cytoplasmic side of the membrane; out, cell wall-exposed side of the membrane.

these phenotypes changed from wild-type growth, or near wild-type growth, to a severe or very severe phenotype. This strongly suggests TatAc associates with TatAy with regards to translocating active EfeB, and that the residues P2, P21, A31 and G32 are of particular importance for this interaction. Notably, TatAc on its own is unable to compensate for the complete absence of the TatAy or TatAd proteins (134). However, when TatAc was re-introduced into the strains with P2A, P2D, P21A, or A31G substitutions in TatAy, it fully restored growth to wild-type rates indicating wild-type EfeB translocation and activity.

Two different phenotypes were observed with regards to different substitutions for the proline residue at position two of TatAy (i.e. P2A and P2D). This residue is conserved in the TatA-like proteins of Gram-positive bacteria, but not in Gram-negative bacteria (23). A mutation in the equivalent residue of TatAd (TatAd^{P8A}) has been shown in the *E. coli* system to be particularly important with regards to functionally replacing TatB (23). This suggests that this region may be more important in docking complex functioning than pore formation. The mutation TatAy^{P2A}, where proline was replaced by an alanine, was not accepted in any genetic background and severe phenotypes were observed. An aspartic acid at this position however was tolerated, although to a lesser extent in a background where TatAc was absent. Hence the P2 residue could be involved in important TatAy-TatCy interactions, translocation or processing of the substrate, or in TatAy pore formation. After the re-introduction of TatAc into a *tatAc-tatAyCy* mutant background, EfeB translocation and growth phenotypes reverted to wild-type. This suggests that in the presence of TatAy with defective N-terminal regions, wild-type TatAc is able to compensate and fulfil the role of TatAy.

The growth phenotype associated with the G20A substitution in the hinge region of TatAy was distinctive, because it was very severe in all four *tat* mutant backgrounds tested. In contrast, the TatAy P21A substitution only had a very severe growth phenotype in backgrounds without *tatAc*. The reintroduction of ectopically expressed TatAc, however was able to compensate completely for the G20A and P21A mutations in TatAy. This suggests that the amount of TatAc expressed from the complementing plasmid can fully compensate for TatAy with regard to important residues in the hinge region, as in the case of the N-terminal amino acid substitutions.

Mutated residues in the amphipathic helix of TatAy that had disrupted EfeB translocation, namely L24A, A31G and G32A, are highly conserved in other bacterial TatA proteins (82, 86). The residues L24 and A31 are both thought to lie against the membrane while the hydrophilic G32 residue faces the cytoplasm. The growth phenotypes associated with the L24A and A31G

substitutions were reversed when TatAc was reintroduced. Intriguingly, this was not the case for the G32A substitution. It is thus possible that the mutation G32A disrupts an essential TatAy-EfeB interaction (docking complex) or TatAy-TatAy (pore) interaction. The equivalent substitution of TatAy G32A in TatAd is G34A. It was recently suggested that the G34 residue is located in a region of the amphipathic helix involved in intramolecular salt bridges and self-assembly of the TatAd protein (60). Hence the inability of TatAc to compensate for TatAy G32A could be due to a disruption of intramolecular salt bridges. A second theory as to why TatAc is unable to compensate for TatAy G32A is that this residue may be involved in specific EfeB recognition or quality control performed by the TatA-TatC docking complex and that TatAc is unable to compensate for this TatAy mutant protein in the docking complex. According to this theory, TatAy (or potentially TatAd) would need to be present in order for TatAc to actively facilitate substrate translocation. This could lead on to a hypothesis that in *B. subtilis* the mutated TatAy behaves much like TatB in *E. coli.* In this case, TatAc would serve the function of *E. coli* TatA. This would imply that the TatAy mutant proteins of strains which show severe phenotypes have lost their bifunctionality. This is not the case for TatAy G32A, which seems to have lost all functionality.

In conclusion, phylogenetic analyses comparing the Tat components from all kingdoms have suggested that in most cases the TatA system is composed of two TatA-like components and one TatC component (36, 48). In the *E. coli* system these two TatA-like proteins, TatA and TatB, have evolved distinct functions. The work presented here suggests that the third TatA-like protein in *B. subtilis* might represent an intermediate evolutionary step in TatA-TatB specialization. Accordingly, in *B. subtilis* one TatA-like protein (TatAc) could predominantly form part of the pore protein complex for translocation. The second TatA-like protein (TatAd or TatAy) could form a docking complex with TatC to allow for substrate recognition and quality control. Thus, the *B. subtilis* TatA components have not 'devolved' as far from each other as TatA and TatB in *E. coli,* since they are still bifunctional in docking complex and pore formation.

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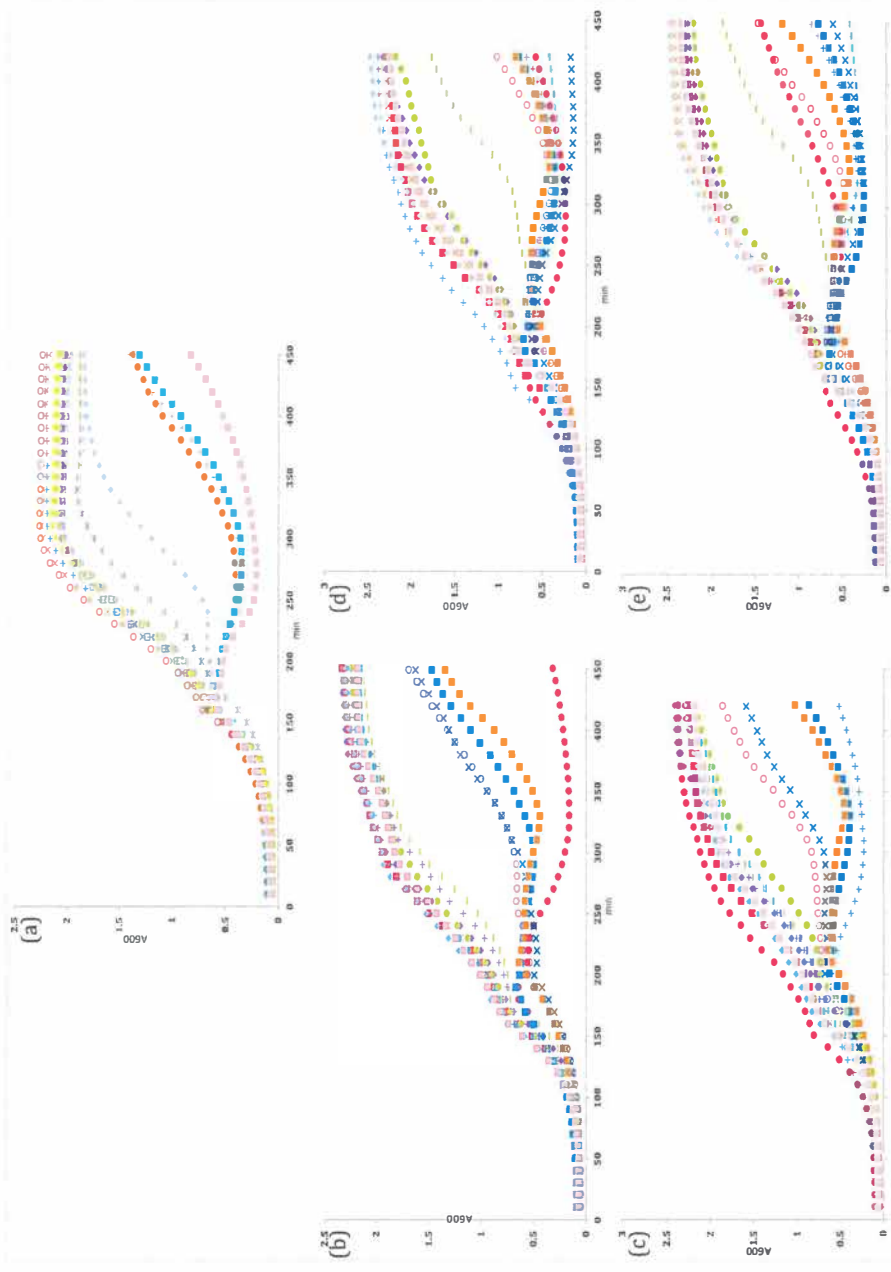
Supplementary Materials



Supplementary Figure 1 (adjacent page). Resumed growth of *tat* mutant strains in LB without NaCl. The genetic backgrounds in the different panels are as follows **(a)** *tatCd-tatCy*, where strains are represented as; *B. subtilis* 168 (•),*tatCd-tatCy* mutant background (•), *tatCd-tatCy* mutant background with pHB-*tatCy*$^{wild\text{-}type}$ (+), pHB-*tatCy*^{L11A} (○),pHB-*tatCy*^{H14A} (–), pHB-*tatCy*^{I15A} (◆), pHB-*tatCy*^{L18A} (■), pHB-*tatCy*^{R19A} (□), pHB-*tatCy*^{K20A} (□), pHB-*tatCy*^{P96A} (×), pHB-*tatCy*^{G97A} (•), pHB-*tatCy*^{L98A} (+), pHB-*tatCy*^{E102A} (–), pHB-*tatCy*^{S108A} (◆), pHB-*tatCy*$^{C\text{-}5}$ (◆), or pHB-*tatCy*$^{C\text{-}8}$ (■). **(b)** *tatAyCy*, **(c)** *tatAdCd-tatAyCy* **(d)** *tatAc-tatAyCy* and **(e)** total-*tat* background, strains in **(b-e)** are represented as followed: *B. subtilis* 168 (■), mutant background detailed in individual panel without any plasmid (•), mutant background with pHB-Ay$^{wild\text{-}type}$Cy$^{wild\text{-}type}$ (+), pHB-Ay^{P2A}Cy$^{wild\text{-}type}$ (○), pHB-Ay^{P2D}Cy$^{wild\text{-}type}$ (–), pHB-Ay^{F19A}Cy$^{wild\text{-}type}$ (◆), pHB-Ay^{G20A}Cy$^{wild\text{-}type}$ (■), pHB-Ay^{P21A}Cy$^{wild\text{-}type}$ (□), pHB-Ay^{K23A}Cy$^{wild\text{-}type}$ (□), pHB-Ay^{L24A}Cy$^{wild\text{-}type}$ (■), pHB-Ay^{G28A}Cy$^{wild\text{-}type}$ (•), pHB-Ay^{A31G}Cy$^{wild\text{-}type}$ (+), pHB-Ay^{G32A}Cy$^{wild\text{-}type}$ (–), pHB-Ay^{F38A}Cy$^{wild\text{-}type}$ (◆), pHB-Ay^{N40A}Cy$^{wild\text{-}type}$ (■).

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Chapter 4

Co-factor insertion, disulphide bonding and Tat translocase requirements for membrane translocation of the *Bacillus subtilis* Rieske protein QcrA

Vivianne J. Goosens, and Jan Maarten van Dijl, submitted to *J. Biol. Chem.*

Abstract

The twin-arginine translocation (Tat) system transports folded and co-factor-containing cargo proteins over bacterial cytoplasmic membranes. Functional Tat machinery components and a folded state of the cargo protein are both fundamental to successful translocation. The present studies dissected these requirements with regard to the Rieske iron-sulphur protein QcrA of *Bacillus subtilis*. Notably, QcrA is a component of the cytochrome bc_1 complex, which is conserved from bacteria to man. Single amino acid substitutions were introduced into the Rieske domain of QcrA to prevent either co-factor binding or disulphide bond formation. Both types of mutations precluded QcrA translocation. Additionally, a proofreading hierarchy was uncovered, where a QcrA mutant defective in disulphide bonding was quickly degraded, while mutant QcrA proteins defective in co-factor binding accumulated in the cytoplasm and membrane. Further, studies investigating QcrA translocation by site-specifically mutated Tat machinery components known as TatAy and TatCy were performed in various genetic backgrounds and under different culturing conditions. These analyses revealed residues in the amphipathic helix of *B. subtilis* TatAy, which are involved in cargo-specific interactions. Importantly, these are the first studies on Tat-dependent protein translocation where both oxidative folding and co-factor attachment have been addressed in a single native molecule.

Introduction

The movement of proteins from the inside of cells over or into a membrane is of vital importance with regards to many biological processes, such as nutrient uptake, metabolism, respiration, and cell-cell communication. To facilitate the transport of proteins across the bacterial cytoplasmic membrane, various protein export mechanisms have evolved. The main protein export pathway relies on the membrane-embedded Sec translocation machinery, which translocates proteins in an unfolded state across the cytoplasmic membrane. However, other more specialized mechanisms, such as YidC-facilitated membrane protein insertion or the twin-arginine translocation (Tat) system also make important contributions to bacterial protein export from the cytoplasm (1, 2, 4, 7).

The Tat pathway is unique in that it is able to translocate correctly folded (15, 16, 18) and cofactor-attached cargo proteins (50, 91, 96). Apart from the globular nature of the Tat-dependent cargo, a second distinctive

feature of the Tat system is that of the eponymous twin-arginine motif within its signal peptide (11, 14, 35). The current interpretation of the mechanism associated with Tat translocation is that the cargo proteins are first recognized by a docking complex composed of TatC and TatA-like proteins (38, 39). Importantly, it is thought that the cargo protein undergoes proofreading and quality control at this point (11, 40-43). After docking complex and cargo protein have interacted, more TatA-like components are recruited such that multiple TatA-like components form a translocation pore (40). The proton-motive force then drives translocation (45, 46).

Bacillus subtilis is a non-pathogenic Gram-positive bacterium with the generally recognized as safe (GRAS) status. Due to its high capacity for protein secretion, *B. subtilis* is not only widely applied in biotechnology, but it has also become an important model organism for fundamental studies on protein export and secretion (12-14). The Tat system in *B. subtilis* is composed of three TatA-like proteins (TatAd, TatAy and TatAc) and two TatC proteins (TatCd and TatCy). TatAd-TatCd and TatAy-TatCy combine to from two independent parallel protein transport pathways, each with its own substrate specificities (72). Although the third TatA component, TatAc, is unable to functionally replace the other TatA components (134), recent studies have uncovered a role for TatAc in assisting TatAy during translocation (Chapter 3). The *tatAy-tatCy* operon is expressed under a broad range of conditions, which corresponds to the broad expression profile of the known substrates of the TatAy-TatCy translocase, namely the Dyp-type peroxidase EfeB (YwbN), the alkaline phosphatase YkuE, and the Rieske iron-sulphur protein QcrA (also known as PetC) (20, 72, 129).

Rieske iron-sulphur proteins - in short Rieske proteins - form part of the cytochrome bc_1 complex in *B. subtilis.* Cytochrome bc_1 is a main component of the electron transport chain and, as a homolog of the complex III in mitochondria, it is structurally and functionally highly conserved in all three kingdoms of life (230, 231). In *B. subtilis*, the cytochrome bc_1 complex is a menaquione:cytochrome *c* reductase composed of the Rieske protein (QcrA), cytochrome *b* (QcrB) and a larger than normal cytochrome c_1 (QcrC) (186). Notably, cytochrome bc_1 is a membrane protein complex that faces the extracellular side of the cytoplasmic membrane. The Sec pathway individually translocates the cytochrome *b* and cytochrome c_1 components across the membrane before they are processed, matured and combined (173, 230, 232-235). It is believed that cytochrome *b* and cytochrome c_1 form a primary protease-resistant complex, where the final step in complex maturation is the incorporation of the Rieske protein (230).

All Rieske proteins contain a well-defined Rieske domain. Within this domain there are specific residues involved in 2Fe-2S co-factor binding. In some Rieske proteins, like QcrA of *B. subtilis*, the Rieske domain contains Cys residues involved in disulphide bond formation (126-128, 231). Apart from QcrA of *B. subtilis,* other Rieske proteins also share a strong association with the Tat pathway as was demonstrated in various bacteria and chloroplasts of green plants (21, 114-116). Notably, the Gram-negative bacterium *Escherichia coli* does not contain a cytochrome bc_1 complex, which precludes studies on Rieske protein assembly in this important model organism for research on Tat-dependent protein translocation (230, 231). *B. subtilis* on the other hand is an attractive organism to explore the requirements and Tat-dependency of Rieske protein translocation. Firstly, the electron transport chain in *B. subtilis* has two branches, creating a functional redundancy that allows for cytochrome bc_1 mutation studies (230, 236, 237). Secondly, the *qcrABC* operon is highly expressed under a wide range of tested conditions and, consequently, it does not need to be artificially induced to investigate QcrA export (130, 131). The present studies were therefore aimed at analysing the requirements for Tat-dependent export of QcrA in *B. subtilis*. This involved site-directed mutagenesis of both QcrA and Tat components. QcrA was mutated to assess the roles of co-factor assembly and disulphide bonding in membrane translocation. Notably, specific amino acid residues in the *B. subtilis* TatAy and TatCy proteins were previously shown to be fundamental for the translocation of EfeB ((68, 83) and Chapter 3). Therefore, the relevance of these residues was also assessed for membrane translocation of QcrA. Lastly, the growth medium salinity has been shown to affect the Tat-dependent export of EfeB (135, 136). Accordingly, the QcrA translocation was examined at various NaCl concentrations. Altogether, these studies reveal a hierarchy in the folding events needed for productive QcrA translocation and pinpoint TatAy residues with substrate-specific roles. To our knowledge this is the first example of an investigation into the importance of folding and co-factor assembly for Tat-dependent membrane translocation of a native, non-engineered cargo protein.

Materials and Methods

Strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Lysogeny Broth (LB) was composed of 1% tryptone, 0.5% yeast extract and, depending on the assay specified in the Results section, 0%, 1% or 6% NaCl. Bacterial cultures were grown in LB at 37 °C under vigorous shaking, or on LB agar plates incubated at 37 °C. *B. subtilis* cells were made competent in

Paris Medium (PM) (168). When appropriate, the cultures were supplemented with antibiotics: *E. coli* cultures with 100 µg/ml ampicillin (Ap) and *B. subtilis* cultures with 2 µg/ml erythromycin (Em), 5 µg/ml chloramphenicol (Cm), 10 µg/ml tetracycline (Tc), 100 µg/ml spectinomycin (Sp), or 20 µg/ml kanamycin (Km).

Table 1. Strains and plasmids used in this study

Strains	Relevant properties	Ref
B. subtilis 168	trpC2	(185)
B. subtilis 168 *tatAdCd*	trpC2, *tatAd-tatCd*::Km; Km^r	(25)
B. subtilis 168 *tatAyCy*	trpC2, *tatAy-tatCy*::Sp; Sp^r	(72)
B. subtilis 168 *tatAc$_1$-tatAyCy*	*trpC2*; *tatAc*::Em, *tatAy-tatCy*::Sp; Em^r; Sp^r	(25)
B. subtilis 168 tatCd-tatCy	*trpC2*; *tatCd*::Km; *tatCy*::Sp; Km^r; Sp^r	(132)
B. subtilis 168 *total-tat$_2$*	trpC2, *tatAd-tatCd*::Km, *tatAy-tatCy*::Sp; tatAc::Em^r; Sp^r, Km^r, Em^r	(72)
B. subtilis qcrA	*trpC2, qcrA*::pPP435	(20)
Plasmids		
pHB-201	*B. subtilis-E. coli* expression vector; ori-pBR322; ori-pTA1060; *cat86::lacZa*; Em^r; Cm^r	(239)
pHB-*AyCy*	pHB201-derivative carrying the *tatAy-tatCy* operon; Em^r; Cm^r	(136)
pHB-TatC^{aa}	pHB201-derivatives carrying *tatCy* genes that specify mutant TatCy proteins with specific single amino acid substitutions or C-terminal deletions; Em^r; Cm^r	(68)
pHB-TatA^{aa}TatCy^{WT}	pHB201-derivatives carrying a wild-type copy of the *tatCy* gene plus *tatAy* genes that specify mutant TatAy proteins with specific single amino acid substitutions; Em^r; Cm^r	(83)
pHB-QcrA	pHB201-derivative carrying wild-type *qcrA*; Em^r; Cm^r	(20)
pHB-QcrA^{H102L}	pHB201-derivative carrying a mutant *qcrA* gene that specifies QcrA-H102L; Em^r; Cm^r	This study
pHB-QcrA^{H124L}	pHB201-derivative carrying a mutant *qcrA* gene that specifies QcrA-H124L; Em^r; Cm^r	This study
pHB-QcrA^{C123S}	pHB201-derivative carrying a mutant *qcrA* gene that specifies QcrA-C123L; Em^r; Cm^r	This study

Cloning and DNA techniques

Cloning and ligation reactions were performed as described previously (238) using products from New England Biolabs. PCR reactions were performed using the Phusion (New England Biolabs) or Pwo (Roche) polymerases. The methodology for site-directed mutagenesis has been described previously in

detail (83). Briefly, though external primers were the same for constructing all *qcrA* mutants, the internal primers included nucleotide changes that translated into single amino acid mutations in the full QcrA sequence. External primers included sequences specifying restriction sites for *Bam*HI and *Spe*I, which allowed for cloning into the *E. coli-B. subtilis* shuttle vector pHB-201. Primers are listed in Table 2.

Table 2. Primers used in this study

External	**Primer sequence**	**Underlined region**
QcrALnew	CGGAAGGTTACTAGTAGGGGTGACTTAGAGGGGG	*Spe*I
QcrARnew	CCG GGATCC GTGTAATATCAAGCCGCTCG	*Bam*HI
Internal		
QcrAH102L-Left	GTACACCCTAAAAGCTTACAAATTG	**CAT→CTT** (His→Leu)
QcrAH102L-Right	CAATTTGTAAGCTTTTAGGGTGTAC	**CAT→CTT** (His→Leu)
QcrAH124L-left	CCGTAAAGGCATGGACAAAAGAATTTAT	**CAT→CTT** (His→Leu)
QcrAH124L-right	ATAAATTCTTTTGTCCATGCCTTTACGG	**CAT→CTT** (His→Leu)
QcrAC123S-left	CCGTAATGGGATGGACAAAAGAATTTAT	**GCC-CCC** (Cys→Ser)
QcrAC123S-right	ATAAATTCTTTTGTCCATCCCATTACGG	**GCC-CCC** (Cys→Ser)

Crude cell fractionations

Crude cell protein extractions were performed after cultures were grown to early stationary phase. Culture aliquots (2 ml) were treated with Complete protease inhibitor (Roche) and pelleted. The extracellular fraction (1.5 ml) was removed and proteins in this fraction were precipitated with TCA overnight. The cell pellet was resuspended in 100 µl lithium dodecyl sulphate (LDS) gel loading buffer and reducing agent (NuPAGE, Invitrogen) before disruption by bead-beating three times with glass beads at 6500 rpm for 3 s with 30 s intervals (Precellys 24 lysis & homogenization, Bertin Technologies). The TCA-precipitated extracellular fraction was acetone washed and resupended in 50 µl LDS gel loading buffer and reducing agent. Samples were heated at 95°C and, if necessary, stored at -20 °C. Crude cell extract aliquots of 10 µl and growth medium aliquots of 20 µl corresponding to 2 OD_{600} units were used for NuPAGE and Western blotting.

Subcellular fractionations

For subcellular fractionation studies, all cultures were grown to early stationary phase. Cells were harvested by centrifugation at 4000 rpm at 4 °C. Then, 1 ml of the supernatant (i.e. the extracellular fraction) was removed and precipitated with TCA overnight. The cell fraction was resuspended in protoplast buffer (100 mM Tris, 20 mM $MgCl_2$, 20 % sucrose, 1 mg/ml lysozyme, 0.01 % DNase, Complete protease inhibitor [Roche]) and incubated at 37 °C for 30 min. The liberated cell wall fraction was thereafter separated from the protoplasts by centrifugation at 4000 rpm at 4 °C. The protoplast supernatant (i.e. the cell wall fraction) was removed and the protoplasts were resuspended in disruption buffer (50 mM Tris pH 8.0, 2.5 mM EDTA) before being disrupted by bead beating three times at 6500 for 30 s with 30 s pauses. Samples were centrifuged at 10,000 rpm to separate glass beads from cytoplasmic content and membranes. The supernatant was then centrifuged at 200,000 g and the supernatant (i.e. the cytoplasmic fraction) was collected. The membrane pellet was resuspended in solubilisation buffer (20 mM Tris, 10 % glycerol, 50 mM NaCl, 0.03 % DDM) and left at 4 °C overnight. Non-solubilized membranes were pelleted by centrifugation at 100,000 g and the supernatant with solubilized membrane proteins (i.e. the membrane fraction) was collected. The protein concentration of samples was estimated using the DC™-Biorad Assay (Biorad) and samples were stored at -20 °C till further use. Aliquots of 10 µg protein were used for sample analysis by NuPAGE and Western blotting.

Gel Electrophoresis and Western Blotting

Proteins were separated using NuPAGE gels (Invitrogen) and transferred onto nitrocellulose membranes (Protran, Schleicher&Schuell) by semi-dry blotting. Polyclonal antibodies specific for BdbD, LipA, QcrA and TrxA have been described previously (20, 180, 240). Bound antibodies were detected with fluorescent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit from LiCor Biosciences) and visualized at 700 or 800 nm with the Odyssey Infrared Imaging System (LiCor Biosciences).

Results

Proofreading hierarchy with regard to co-factor binding and disulphide bond formation.

The Rieske domain is highly conserved in all known Rieske iron-sulphur proteins and, therefore, the residues responsible for both the formation of the disulphide bond and those associated with co-factor attachment are well defined (Figure 1a). To assess the roles of co-factor binding and disulphide bonding in QcrA export in *B. subtilis*, site-specific mutations were introduced into the Rieske domain of QcrA. These involved single amino acid changes altering the co-factor insertion site (H102L or H124L), or a cysteine residue involved in disulphide bond formation (C123S). The *qcrA* genes specifying these amino acid mutations were cloned into the pHB-201 expression plasmid and introduced into a *B. subtilis* strain with a mutated chromosomal *qcrA* gene.

As previously shown by subcellular fractionation, the intact QcrA is an 18-kDa membrane-associated protein with N_{in}-C_{out} topology (20). Importantly, the translocated QcrA is exposed to signal peptidase activity, resulting in the release of a 14-kDa processed form (QcrA*) into the growth medium (Figure 1b, and Chapter 2). As the catalytic site of signal peptidase is positioned on the extracytoplasmic side of the membrane, QcrA processing and release of QcrA* into the medium can be used as a read-out for the translocation of this protein. Hence, the membrane translocation of QcrA was visualised by Western blotting of crude whole-cell extracts and growth medium fractions. As shown in Figure 1b, QcrA* was absent from the growth media of all strains expressing mutated forms of QcrA (C123S, H102L or H124L). Interestingly, in the crude whole-cell extract, the presence of full-size QcrA was observed only for the co-factor-binding site mutants (H102L and H124L) and the wild-type protein, but not for the mutant impaired in disulphide bonding (C123S).

All three mutant QcrA proteins are expressed with the same vector and, therefore, the absence of QcrA-C123S implies fast degradation of this non-disulphide bonded protein. This is in line with the previously documented degradation of other extracytoplasmic proteins, such as ComEC and ComGC, lacking an essential disulphide bond (171, 172). It should however be noted that, unlike ComEC and ComGC, the presence of QcrA was not affected by a *bdbC-bdbD* mutation (Figure 1c), suggesting that oxidative folding of QcrA is independent of the BdbC-BdbD thiol-disulphide oxidoreductases (TDORs).

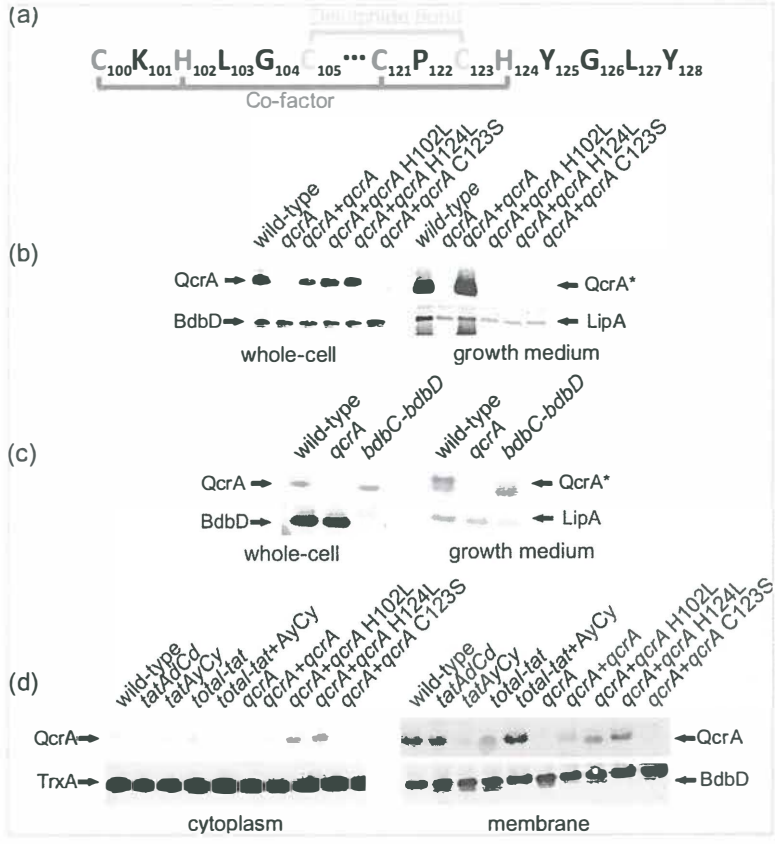


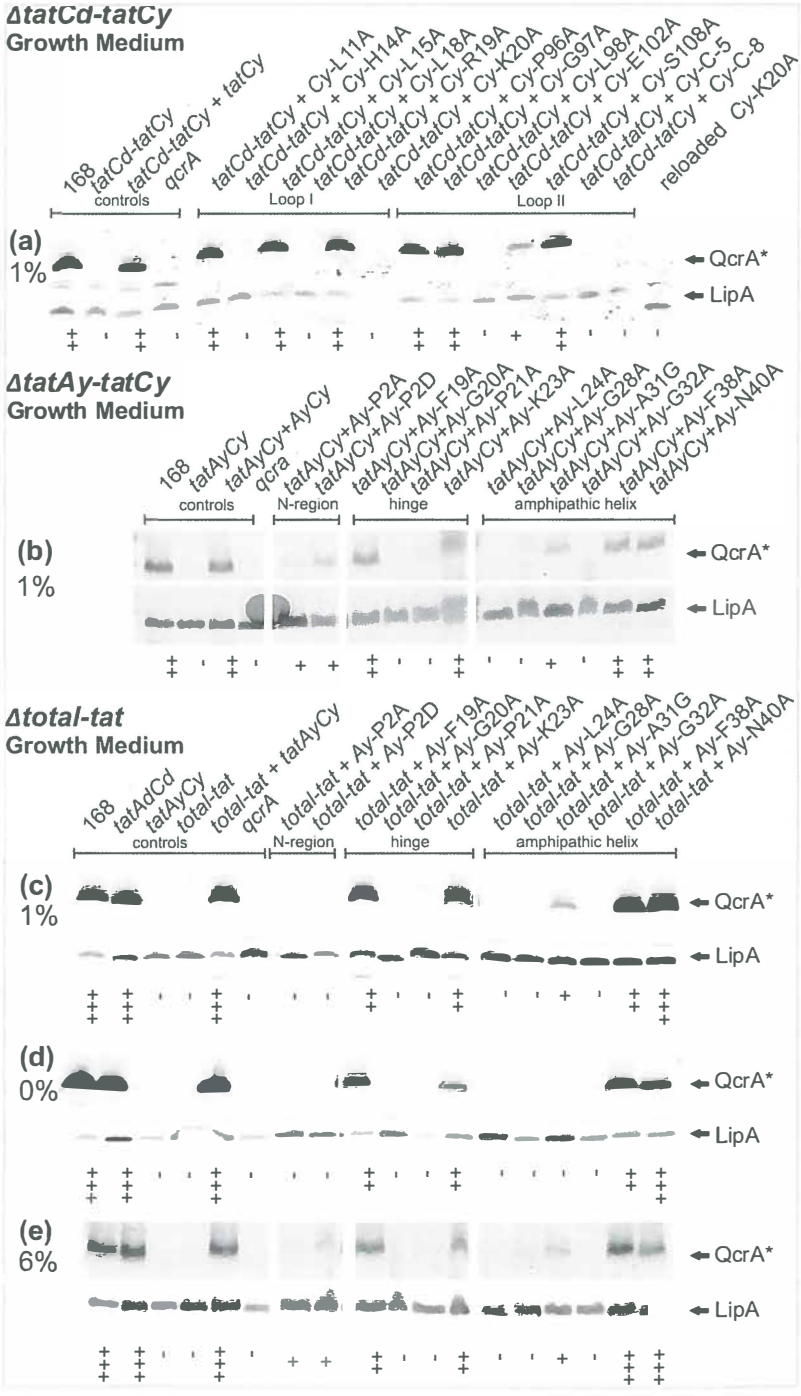
Figure 1. Translocation and quality control of QcrA mutant proteins defective in co-factor-binding and disulphide bond formation (a) The Rieske domain of *B. subtilis* QcrA with annotated amino acid residues involved in co-factor attachment and disulphide bond formation. **(b)** Translocation of QcrA in strains with a deleted chromosomal *qcrA* gene that ectopically express wild-type *qcrA* or site-specifically mutated *qcrA* genes. The mutant *qcrA* genes specified the H102L or H124L mutant QcrA proteins impaired in co-factor-binding, or the C123S mutant QcrA protein defective in disulphide bond formation. All strains were grown in LB with 1% NaCl. Cells were separated from the growth medium, and crude whole-cell extracts and growth medium fractions were analysed by Western blotting using specific antibodies against QcrA, BdbD, or LipA. The positions of the 18 kDa full-size QcrA protein in the whole-cell fraction and the 14 kDa processed QcrA protein (QcrA*) in the growth medium fraction are marked with arrows. The membrane protein BdbD and the secreted protein LipA were used as positive controls. **(c)** Translocation of QcrA by *bdbC-bdbD* mutant cells was assessed as described for panel a. **(d)** Subcellular fractionation was performed to separate the cytoplasmic and membrane proteins of *tat* mutant strains and *qcrA* mutant strains producing site-specifically mutated QcrA proteins as indicated. The positions of QcrA, the cytoplasmic marker protein TrxA and the membrane protein BdbD are marked with arrows.

Strains producing mutated QcrA proteins were further investigated by subcellular fractionation. Samples were taken from cultures in the early stationary phase. Mutant proteins defective in the co-factor-binding site (QcrA-H102L or -H124L) were detectable in the membrane fraction (Figure 1c). As signal peptidase-processed forms of QcrA-H102L and QcrA-H124L were completely absent from the growth medium (Figure 1a), these findings imply that correct co-factor-binding by QcrA is a prerequisite for productive membrane translocation. In contrast, the QcrA-C123S mutant protein defective in disulphide bond formation was neither observed in the cytoplasmic, nor the membrane or the extracytoplasmic fractions. This supports the view that a QcrA mutant protein with the inability to form a correct disulphide bond is degraded shortly after synthesis and does neither build up in the cytoplasm nor reach the membrane. Together, these observations imply that there is a proofreading hierarchy where non-disulphide bonded QcrA is rapidly degraded, while QcrA defective in co-factor binding is stable.

Mutations in TatAy or TatCy affecting QcrA translocation.

Previous studies have shown that individual amino acid residues in TatAy or TatCy are essential for the active translocation of the TatAy-TatCy-dependent cargo protein EfeB (Chapter 3 of this thesis and (68, 83)). The importance of these residues in TatAy and TatCy was therefore investigated with regard to QcrA translocation by examining the presence of processed QcrA (i.e. QcrA*) in growth medium fractions. QcrA expression is highest in the early stationary growth phase (130, 131) and, therefore, samples were taken at this growth stage.

Figure 2 (adjacent page). Translocation of QcrA in *tat* mutant strains (a) Release of processed QcrA (QcrA*) into the growth medium of strains lacking the chromosomal *tatCd* and *tatCy* genes and grown in LB with 1% NaCl as detected by Western blotting. The *tatCd-tatCy* mutant strains were complemented with ectopically expressed wild-type *tatCy* or a range of site-specifically mutated *tatCy* genes as indicated for each lane. The presence or absence of QcrA* is indicated with +/- underneath the Western blot, +++ indicating the highest QcrA* level and + the lowest. The sample of the strain expressing TatCy-K20A was re-loaded to clarify the absence of QcrA*. The secreted LipA protein was used as a positive control. **(b)** Release of QcrA* into the growth medium of strains lacking the chromosomal *tatAy-tatCy* operon and grown in LB medium with 1% NaCl as detected by Western blotting. The *tatAy-tatCy* mutant strains were complemented with ectopically expressed wild-type *tatAy-tatCy* or *tatAy-tatCy* operons encoding wild-type *tatCy* plus site-specifically mutated *tatAy* genes as indicated. The presence or absence of QcrA* is indicated with +/- underneath the Western blot as in panel a. **(c-e)** Release of QcrA* into the growth medium of strains lacking all chromosomal *tat* genes (total-*tat*) and grown in LB media with 1% NaCl **(c)**, 0% NaCl **(d)**, or 6% NaCl **(e)** as detected by Western blotting. The total-*tat* strains were complemented with ectopically expressed wild-type *tatAy-tatCy* or *tatAy-tatCy* operons encoding wild-type *tatCy* plus site-specifically mutated *tatAy* genes as indicated. The presence or absence of QcrA* is indicated with +/- underneath each Western blot as in panel a.


ΔtatCd-tatCy
Growth Medium
168
tatCd-tatCy
tatCd-tatCy + tatCy
qcrA
tatCd-tatCy + Cy-L11A
tatCd-tatCy + Cy-H14A
tatCd-tatCy + Cy-L15A
tatCd-tatCy + Cy-L18A
tatCd-tatCy + Cy-R19A
tatCd-tatCy + Cy-K20A
tatCd-tatCy + Cy-P96A
tatCd-tatCy + Cy-G97A
tatCd-tatCy + Cy-L98A
tatCd-tatCy + Cy-E102A
tatCd-tatCy + Cy-S108A
tatCd-tatCy + Cy-C-5
tatCd-tatCy + Cy-C-8
reloaded Cy-K20A
controls
Loop I
Loop II
(a)
1%
QcrA*
LipA
ΔtatAy-tatCy
Growth Medium
168
tatAyCy
tatAyCy+AyCy
qcra
tatAyCy+Ay-P2A
tatAyCy+Ay-P2D
tatAyCy+Ay-F19A
tatAyCy+Ay-G20A
tatAyCy+Ay-P21A
tatAyCy+Ay-K23A
tatAyCy+Ay-L24A
tatAyCy+Ay-G28A
tatAyCy+Ay-A31G
tatAyCy+Ay-G32A
tatAyCy+Ay-F38A
tatAyCy+Ay-N40A
controls
N-region
hinge
amphipathic helix
(b)
1%
QcrA*
LipA
Δtotal-tat
Growth Medium
168
tatAdCd
tatAyCy
total-tat
total-tat + tatAyCy
qcrA
total-tat + Ay-P2A
total-tat + Ay-P2D
total-tat + Ay-F19A
total-tat + Ay-G20A
total-tat + Ay-P21A
total-tat + Ay-K23A
total-tat + Ay-L24A
total-tat + Ay-G28A
total-tat + Ay-A31G
total-tat + Ay-G32A
total-tat + Ay-F38A
total-tat + Ay-N40A
controls
N-region
hinge
amphipathic helix
(c)
1%
QcrA*
LipA
(d)
0%
QcrA*
LipA
(e)
6%
QcrA*
LipA

The effect of amino acid replacements in TatCy on QcrA translocation was investigated in a strain devoid of both chromosomal *tatC* genes, but expressing a plasmid-borne mutated *tatCy* copy. Specifically, the *tatCy* gene was altered to generate 13 different mutant proteins. These included 11 TatCy proteins with individual amino acid replacements, and two TatCy proteins with C-terminal truncations of 5 or 8 residues, respectively (Chapter 3 and (68). The extracellular QcrA* profiles of these strains showed that the mutations at residues H14A, L18A, K20A, L98A, and the two C-terminal truncations cause QcrA translocation defects (Figure 2a).

The effects of replacements of individual amino acid residues in TatAy were investigated in *tatAy-tatCy* or *total-tat* mutant *B. subtilis* strains. To this end, a plasmid expressing the *tatAy-tatCy* operon was used where the *tatAy* gene carried the respective site-specific mutations. Here, 12 previously described TatAy mutant proteins were assessed for their ability to facilitate QcrA translocation (Chapter 3 and (83)). Assessment of the QcrA* profiles of the respective mutant strains by Western blotting showed that the effects of specific TatAy mutations in the *tatAy-tatCy* and *total-tat* backgrounds were very similar (Figure 2, b and c). However, a slight variation was observed, where the TatAy-P2A and TatAy-P2D mutations in the N-terminal region of TatAy both allowed for a small amount of QcrA translocation in the *tatAy-tatCy* background (Figure 2b), but not in the total-*tat* mutant background (Figure 2c). Furthermore, amino acid replacements in the hinge region of TatAy (i.e. G20A and P21A), and in the amphipathic helix of at TatAy (i.e. L24A, G28A and G32A) precluded the appearance of extracellular QcrA*, irrespective of the *tatAy-tatCy* or the *total-tat* mutant backgrounds in which these mutant proteins were expressed. This implies that the respective TatAy mutant proteins are unable to facilitate QcrA translocation.

Effects of growth medium salinity on QcrA translocation

The Tat-dependency of the *B. subtilis* EfeB protein is altered by environmental salinity, and studies with TatAy mutant proteins suggested that this could be related to an intrinsic salt sensitivity of the Tat translocase (83, 135, 136). Therefore, the translocation of QcrA in various *tat(Ay)* mutant strains of *B. subtilis* was investigated upon growth in LB containing 0% (no NaCl), 1% NaCl (normal salt concentration) or 6% NaCl (high salt). Interestingly, the profiles of QcrA* in early stationary phase cultures of strains producing wild-type or TatAy mutant proteins showed that QcrA translocation remained TatAy-TatCy-dependent at all tested salt concentrations (Figure 2, panels c-e; please note that only the results obtained for the total-*tat* mutant background are shown in panels d and e as the results obtained for the total-*tat* mutant and the *tatAy-tatCy* mutant backgrounds were essentially the

same). Altogether, these findings show that QcrA translocation is not drastically affected by environmental salinity. However, upon growth in LB with 6% salt small amounts of QcrA* were released by strains expressing the P2A or P2D mutant TatAy proteins, while these strains released no QcrA* when grown in LB with 1% salt or no salt. Importantly, upon examination of the crude whole-cell extractions of the strains producing mutant TatCy or TatAy proteins, a build-up of QcrA was observed for all investigated strains with an intact *qcrA* gene (Figure 3; only the results obtained from cells grown in LB with 1% NaCl are shown). This build-up was indicative of QcrA production, and it clearly showed that *qcrA* expression was not impaired in any of the investigated strains that were unable to release QcrA* into the medium.

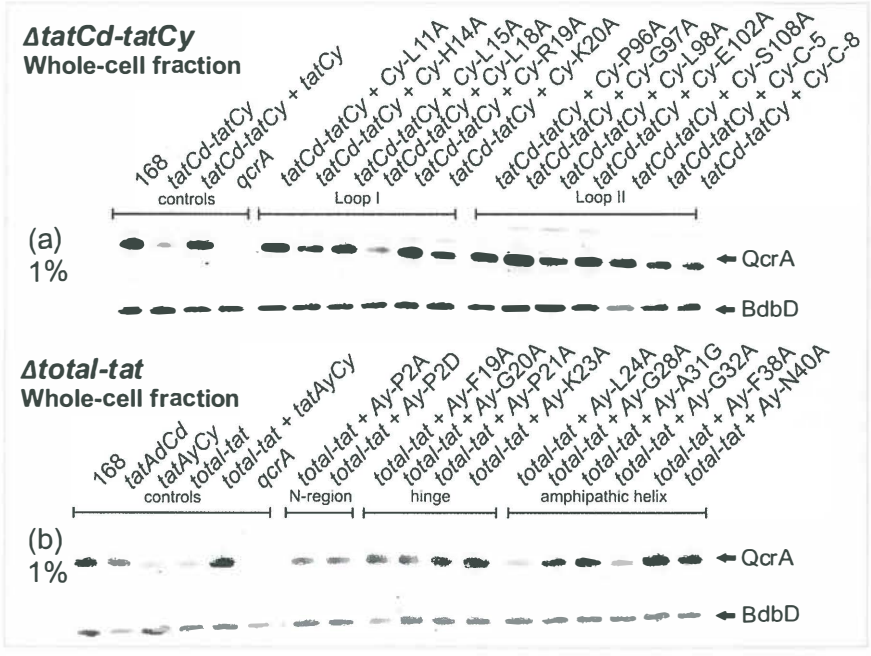


Figure 3. Production of QcrA in *tat* mutant strains (a) The presence of full-size QcrA in whole-cell extracts of strains lacking the chromosomal *tatCd-tatCy* genes and grown in LB medium with 1% NaCl as detected by Western blotting. The *tatCd-tatCy* mutant strains were complemented with ectopically expressed wild-type *tatCy* or different site-specifically mutated *tatCy* genes as indicated. The BdbD protein was detected as a positive control. **(b)** The presence of full-size QcrA in whole-cell extracts of strains lacking all chromosomal *tat* genes (total-*tat*) and grown in LB medium with 1% NaCl as detected by Western blotting. The *total-tat* strains were complemented with ectopically expressed wild-type *tatAy-tatCy* or *tatAy-tatCy* operons encoding wild-type *tatCy* plus site-specifically mutated *tatAy* genes as indicated. The BdbD protein was detected as a positive control.

Discussion

The Tat system is unique in that it translocates folded and co-factor-containing cargo proteins over the cytoplasmic membrane (9, 11, 35). There are a number of known requirements for successful Tat-dependent translocation of cargo proteins. These include a cargo protein with an associated twin-arginine signal peptide, a properly folded state of the cargo protein, and a functional Tat pathway (9, 11, 35). In the present studies, the latter two requirements were investigated for the Tat-dependent membrane translocation of the Rieske protein QcrA of *B. subtilis*. Single amino acid substitutions were introduced into QcrA, which either preclude the oxidative folding of this protein or the insertion of its 2Fe-2S co-factor. In this manner, the importance of folding and co-factor insertion for the productive translocation of QcrA was shown. Additionally, the functionality of components of the Tat-pathway was examined by comparing the translocation of QcrA in strains where either TatAy or TatCy carried site-specific mutations.

Several previous studies have shown that, if cargo proteins of the Tat pathway are not correctly folded or if co-factors are inserted incorrectly, the membrane translocation process is terminated and the cargo degraded (88-92). However, it is noteworthy that studies investigating disulphide bond-assisted folding in the Tat system have, thus far, only been performed on heterologous proteins or with *E. coli* fusion proteins (15, 16, 18, 94, 95). Furthermore, spinach-derived Rieske proteins required a partially folded state to allow for co-factor insertion when expressed in *E. coli* (241). Our present study is the first to examine the Tat-dependent export of a native protein, the Rieske protein QcrA of *B. subtilis*, that requires both co-factor-binding and disulphide bond formation for its biological function. Importantly, the Rieske protein domain is highly conserved and residues involved in disulphide bond formation and co-factor insertion are well defined by high-resolution structural analyses (126-128, 231). On this basis, a total of three site-specific mutations in QcrA were generated; in two mutant proteins the His residues involved in co-factor binding were individually replaced (i.e. QcrA-H102L and QcrA-H124L) and in a third mutant protein a Cys residue involved in disulphide bond formation was replaced (i.e. QcrA-C123S). Membrane insertion and translocation of these mutant QcrA forms was assessed by Western blotting, taking advantage of the fact that translocated QcrA is to some extent processed by signal peptidase, which results in the release of a smaller form (QcrA*) into the growth medium. This clearly showed that all three QcrA mutant proteins were defective in complete translocation. Therefore, both the correct folding and co-factor insertion are critical for Tat-dependent translocation of QcrA. Interestingly, QcrA-C123S was undetectable even in crude cell extracts, indicating that this mutant protein was de-

graded quickly. In contrast, QcrA-H102L and QcrA-H124L were observed in the cytoplasmic and membrane fractions. These findings suggest a sequential and hierarchical quality control process with regard to the Tat-dependent cargo protein QcrA, where oxidative folding undergoes quality control before co-factor insertion. This idea would be supported by the finding that disulphide bond formation in QcrA is independent of the extracytoplasmic TDORs BdbC and BdbD, and it would be consistent with the view that insertion of the 2Fe-2S co-factor takes place in the cytoplasm. If so, this would be the first illustration of a cytoplasmic proofreading or quality control mechanism for a Tat-dependent cargo protein in *B. subtilis*.

It is conceivable that disruption of the disulphide bond in the QcrA-C123S mutant protein caused a structural change that also affected cofactor insertion even though the side-chains of Cys and Ser are relatively similar in size. Therefore, the high instability of the QcrA-C123S protein could relate to defects in both oxidative folding and co-factor binding. Here it is relevant to bear in mind that Rieske proteins are defined by their 2Fe-2S co-factor and the conserved co-factor binding motif. In contrast, not all Rieske proteins have or require the disulphide bond within the co-factor binding motif (231). This suggests that the 2Fe-2S-binding motif may not be dependent on the disulphide bond for co-factor insertion. Unfortunately, it will be extremely hard to further separate the requirements for oxidative folding and co-factor insertion in QcrA of *B. subtilis*, as these processes are interlinked and both important. Moreover, such studies are further complicated by the production of many proteases in *B. subtilis*, which will rapidly degrade folding intermediates of QcrA (112, 242).

Previous studies have examined the importance of individual amino acid residues in both TatAy and TatCy with regard to the translocation of the Tat-dependent EfeB protein. This was done either by Western blotting after xylose-induced expression of an EfeB-myc reporter construct (68, 83), or by grading of the lysis phenotype associated with different deficiency levels in the translocation of active EfeB (Chapter 3). In the current study, the same TatAy or TatCy mutant strains were used to examine translocation of native QcrA by Western blotting. Consistent with the fact that both EfeB and QcrA require the TatAy-TatCy translocase for export, the translocation profile observed for QcrA in strains expressing TatAy amino acid mutants in strains lacking *tatAy-tatCy* or all *tat* genes (i.e. the total-*tat* mutant) were very similar to those observed for EfeB (Chapter 3 and (83)). For example, two amino acid residues in the hinge region of TatAy (Gly20 and Pro21) were found to be important to QcrA translocation in both *tatAy-tatCy* and *total-tat* backgrounds, and these same residues were also important for translocation of active EfeB. On the other hand, for some residues in the amphipathic helix of

TatAy interesting variations were observed. The A31G mutation did not block QcrA translocation, while it severely impaired export on EfeB in the absence of TatAc (Chapter 3). Furthermore, the G28A mutation was found to be essential for QcrA translocation, while it had no apparent effect on the export of active EfeB (Chapter 3). This implies that these amphipathic helix residues may be involved in cargo-specific interactions, i.e. TatAy-G28A with QcrA or TatAy-A31G with EfeB. This view is supported by the findings that the TatAy-G28A mutant was shown to be defective in export of the xylose-induced myc-tagged EfeB reporter (83), which in some TatAy mutant backgrounds behaved differently from the native EfeB expressed from its own promoter (Chapter 3). These cargo-specific relationships might occur at the level of the TatAy-TatCy docking complex, where they could be associated with substrate-specific proofreading mechanisms. However, they could also be associated with pore-formation, or with an as yet unidentified chaperone interaction. Notably, the results obtained with TatCy amino acid mutants showed the same translocation profile previously shown for EfeB-myc (68) and active authentic EfeB (Chapter 3). This further highlights the possibility that the amphipathic helix region of TatAy is a likely region for cargo-specific interactions.

Previous studies showed that the genetic background affected the severity of the lysis phenotype caused by defects in EfeB export (Chapter 3). Importantly this phenotype intensified when TatAc was absent, which revealed that TatAc has a supportive role in the TatAy-TatCy-dependent translocation of EfeB. Therefore, the translocation profile of TatAy amino acid mutants in genetic backgrounds with TatAc (*tatAy-tatCy*) or without TatAc (total-*tat*) was compared. The only difference in QcrA translocation observed for TatAy mutant proteins expressed in *tatAy-tatCy* or *total-tat* backgrounds concerned those TatAy proteins with mutations in the N-terminal region (i.e. P2A or P2D). In the *tatAy-tatCy* background these mutant proteins facilitated the translocation of small amounts of QcrA, whereas this was not the case in the *total-tat* background. This is suggestive of an involvement of TatAc in QcrA translocation, but an involvement of TatAd cannot be ruled out completely as cells grown in LB do express the *tatAd* gene to low levels (136). Also, these differences are not as clearly demarcated as those observed in the EfeB-associated lysis phenotype. On the other hand, the translocation profile in the present study was visualised by Western blotting and, although this technique gives powerful insights into the translocation process, it does not distinguish subtle differences that are readily detectable by the EfeB-associated lysis assay described in Chapter 3. It is therefore currently not completely clear whether or not TatAc has the potential to assist TatAy with regard to QcrA translocation.

The Tat-dependency of EfeB was shown to vary in LB broth with differing NaCl levels (83, 135, 136). Importantly, in LB with 6% NaCl substantial amounts of EfeB-myc were secreted Tat-independently, whereas this was not observed in LB with 1% NaCl or LB without NaCl. However in the present studies, translocation of QcrA was shown to remain TatAy-TatCy-dependent under all tested growth conditions. Nevertheless, some differences were detectable for strains expressing particular TatAy mutant proteins. For example, the strain expressing the TatAy-A31G mutant protein did not release QcrA* into the growth medium when grown in LB without salt. Also, minor amounts of QcrA* where detectable in the growth medium of strains expressing TatAy-P2A or TatAy-P2D in LB with 6% salt, while this was not the case when these strains were grown in LB with 1% salt or no salt. In this case, the QcrA* profile of the total-*tat* mutant grown in LB with 6% looked akin to the profile of the *tatAy-tatCy* mutant strain, suggesting that a possible assistant role of TatAc in QcrA translocation is potentially not as important when cells are grown in a saline environment. Altogether, it can be concluded that, unlike what was previously shown for TatAy-TatCy-dependent EfeB export, the overall Tat-dependency of QcrA is not greatly affected by environmental salinity.

In summary, our present studies show that residues involved in 2Fe-2S co-factor-binding and oxidative folding are essential for productive membrane translocation of the QcrA protein of *B. subtilis*. Interestingly, a proofreading hierarchy with regard to mutated QcrA was uncovered. The inability to form a specific disulphide bond within its Rieske domain caused the immediate degradation of QcrA, most likely in the cytoplasm, while mutant proteins lacking the residues needed for co-factor binding were apparently proofread at the interface of cytoplasm and membrane. Though stably produced, the latter QcrA mutants were not translocated. Additionally, our studies identified residues in the amphipathic helix of TatAy that are involved in cargo-specific interactions with the TatAy-TatCy-dependently translocated QcrA and EfeB proteins. Lastly, these are the first studies in the Tat field where both folding and co-factor attachment have been addressed in a single native molecule. The relevance of these studies on QcrA, the Rieske protein of *B. subtilis*, is underpinned by the important roles of the cytochrome bc_1 complex in oxidative phosphorylation in all three domains of life.

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Chapter 5

Is proteomics a reliable tool to probe the oxidative folding of bacterial membrane proteins?

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Abstract

The oxidative folding of proteins involves disulphide bond formation, which is usually catalysed by thiol-disulphide oxidoreductases (TDORs). In bacteria, this process takes place in the cytoplasmic membrane and other extracytoplasmic compartments. While it is relatively easy to study oxidative folding of water-soluble proteins on a proteome-wide scale, this has remained a major challenge for membrane proteins due to their high hydrophobicity. Here we have assessed whether proteomic techniques can be applied to probe the oxidative folding of membrane proteins using the Gram-positive bacterium *Bacillus subtilis* as a model organism. Specifically, we investigated the membrane proteome of a *B. subtilis bdbCD* mutant strain, which lacks the primary TDOR pair BdbC and BdbD, by gel-free mass spectrometry. In total, 18 membrane-associated proteins showed differing behaviour in the *bdbCD* mutant and the parental strain. These included the ProA protein involved in osmoprotection. Consistent with the absence of ProA, the *bdbCD* mutant was found to be sensitive to osmotic shock. We hypothesize that membrane proteomics is a potentially effective approach to profile oxidative folding of bacterial membrane proteins.

Innovation

Approximately 30% of all genomes are predicted to encode membrane proteins. However, compared to water-soluble proteins, membrane proteins are substantially less studied due to their high overall hydrophobicity. This intrinsic property of membrane proteins makes them notoriously difficult to analyse at a proteome-wide level, and it has especially hampered the identification of specific post-translational modifications. Accordingly, relatively few membrane-associated oxidatively folded proteins have been identified. Here we have investigated whether recent advances in membrane protein extraction techniques, and gel-free mass spectrometry can be applied to identify TDOR-dependent membrane proteins in *B. subtilis*. Importantly, *B. subtilis* produces many different proteases and incorrectly folded proteins are therefore rapidly degraded. Hence the absence of certain proteins from a mutant lacking the main membrane-associated TDORs BdbC and BdbD was regarded as indicative of BdbCD-dependence or association with BdbCD-dependent proteins. The changes observed in the membrane proteome of *bdbCD* mutant cells reveal novel and unanticipated links between TDOR activity and membrane-associated proteins.

Introduction

The oxidative reaction necessary for disulphide bond formation can occur spontaneously. However, efficient disulphide bond formation between the correct cysteine residues *in vivo* is catalysed by specific enzymes known as thiol-disulphide oxidoreductases (TDORs) (5). In the Gram-positive bacterium *Bacillus subtilis* thiol oxidases have been identified that are referred to as *Bacillus* disulphide bond proteins (Bdb) (5). These TDORs are of critical importance in the application of *B. subtilis* as a cellular factory for secreted proteins with disulphide bonds.

Four Bdb proteins have been identified in *B. subtilis*, namely BdbA, B, C and D. The genes for these proteins are grouped in pairs on the genome: *bdbA* and *bdbB* are found in the Spβ prophage region, while *bdbC* and *bdbD* form an operon on the core genome (5). BdbC and BdbD form a redox pair important for oxidative folding of the competence proteins ComEC (172) and ComGC (5), while BdbB and BdbC are connected to the correct folding of the Spβ prophage-encoded bacteriocin sublancin 168 (5). Apart from these proteins no further native TDOR substrates have been identified in *B. subtilis*. However, both BdbC and BdbD are needed for the heterologous secretion of the alkaline phosphatase PhoA of *E. coli* in an active and protease-resistant state (5).

The available data imply that BdbC and BdbD make up the primary oxidative TDOR unit in *B. subtilis* and, consistent with this view, the *bdbC* and *bdbD* genes are expressed throughout the cell cycle under a wide range of physiologically and industrially relevant conditions (131). The fact that the expression of *bdbC* and *bdbD* is not specific for cells that are competent for genetic transformation suggests the possible existence of BdbC and BdbD substrates that are not associated with competence. However, despite extensive molecular biological and proteomics analyses, no such substrates were identified in the cell wall or spent culture media of *B. subtilis* (unpublished observations). This suggested that particular membrane proteins might be substrates for oxidative folding by BdbC and BdbD.

The primary objective of the present studies was to investigate whether membrane proteomics approaches can be applied to identify membrane proteins of *B. subtilis* that are produced in a BdbCD-dependent manner. Specifically, the membrane proteome of a *B. subtilis bdbCD* mutant strain was analysed by mass spectrometry (MS) and compared to the membrane proteome of the parental *B. subtilis* 168 strain. Notably, the extracytoplasmic compartments and growth medium of *B. subtilis* are highly proteolytic due to the production of a large number of cell wall-associated and extracellular proteases (184). A potential BdbC-BdbD substrate would incorrectly fold in

the absence of these TDORs and therefore become a readily degradable target for these proteases (5, 168). This degradation could potentially also extend to the interacting partners of BdbC and BdbD substrates. Therefore, we considered the absence of particular proteins upon mutation of *bdbCD* as an indicator for potentially direct or indirect Bdb relationships.

Results and Discussion

Mass spectrometric identification of changes in the membrane proteome of bdbCD mutant cells

In the present studies the membrane proteomes of two strains, a *B. subtilis* double mutant (*bdbCD*) devoid of BdbC and BdbD and its parental strain 168, were analysed by gel-free LC-MS/MS and the identified proteins were subsequently compared. Quality of the fractionation was assessed on the basis of different protein banding patterns upon SDS-PAGE (Figure 1A). The absence of BdbD from the membrane of the *bdbCD* mutant strain was confirmed by Western blotting (Figure 1B). Extraction of membrane proteins from *B. subtilis* 168 and the *bdbCD* strain was performed twice, generating two biological replicate experiments. Each sample was injected three times, thereby generating three technical replicates per biological replicate. To confirm the presence of a single protein a minimum of two unique peptides of this protein were needed. Taking these constraints into account, a total number of 681 membrane-associated proteins were identified in our MS runs, of which 43 % were predicted to contain transmembrane domains (Supplementary Table 1). In order to consider a particular protein a lead for BdbCD-dependence it had to be identified in both biological replicates.

The localization of identified proteins was predicted by comparing the results from six different membrane protein prediction algorithms. The number of algorithms predicting whether a protein is localized to the membrane is given in Table 1. No reliable programs predicting protein folds and disulphide bond formation are available as yet. Therefore, for the purpose of our studies, we only determined the number of cysteines and the presence of at least one cysteine was considered suggestive of a potential for disulphide bond formation.

Comparisons between the *B. subtilis* 168 and the *bdbCD* mutant membrane proteomes showed that the majority of the proteins observed were identified in both strains, however a subset of 18 proteins listed in Table 1 showed reproducible variation. Specifically, 15 proteins present in at least two of the *B. subtilis* 168 biological replicates were not identified in the

samples of the *bdbCD* mutant strain. As expected, BdbD was found to be absent from the *bdbCD* mutant, thereby serving as an unambiguous internal standard. BdbC was not identified in the 168 strain, but this can be explained by the fact that BdbC has four transmembrane domains and relatively small cytoplasmic/extracytoplasmic domains. Of the 15 membrane-associated proteins missing from the *bdbCD* strain, the following were predicted to be membrane-associated: the β-glucoside permease BglP, the cysteine transporter TcyP, the minor signal peptidase SipU, the lipoprotein LytA, and the protein of unknown function YxaI. Other proteins included the glutamate-5-semialdehyde dehydrogenase ProA, the putative glycerate kinase GlkX, 5 pyrimidine metabolism-related proteins (PyrAA, PyrAB, PyrH, PyrE, PyrF), the sensor kinase DegS, and the protein YbxA which is linked to an ABC transporter of unknown function. Three proteins were detected in the *bdbCD* strain, but not in the parental 168 strain and were suggestive of BdbCD compensatory mechanisms. They included ResD, the NADPH-cytochrome P450 reductase CypD, and the transcription regulator MsmR.

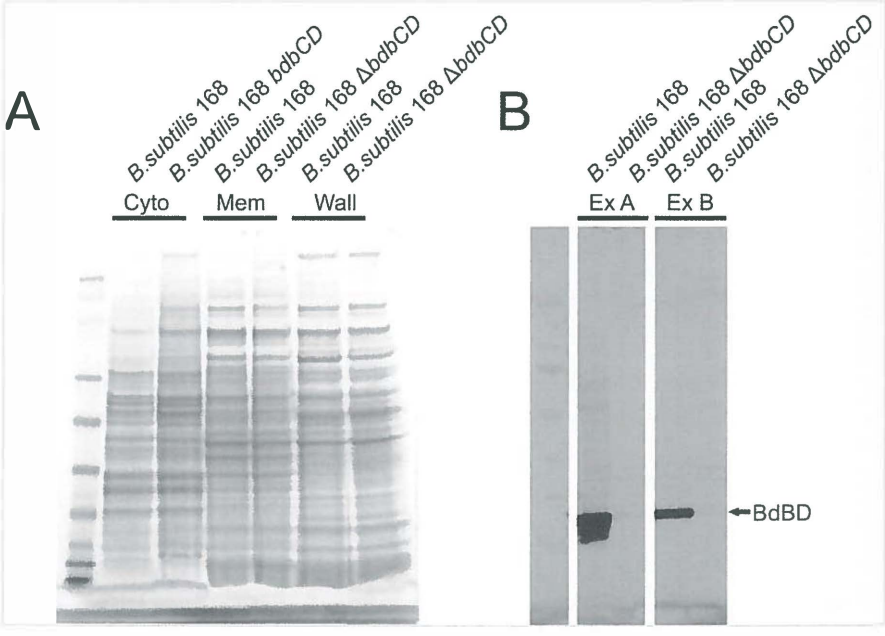

Figure 1. Subcellular fractionation of *B. subtilis*. Cells were fractionated and the quality of the fractionation was subsequently assessed on the basis of different protein banding patterns upon SDS-PAGE. **(A)** Cytoplasmic (Cyto), membrane (Mem) and cell wall (Wall) fractions were collected from cells of a *B. subtilis bdbCD* mutant strain (*bdbCD*) or the parental strain 168 (168) as described in the Materials and Methods. Next, the proteins in these fractions were separated by SDS-PAGE. **(B)** The absence of BdbD from membrane fractions that were used for proteomics analyses was verified by Western blotting with BdbD-specific antibodies. Molecular weight (Mw) markers are indicated (in kDa).

Table 1. Differences between the membrane proteomes of a *B. subtilis* 168 *bdbCD* mutant strain and the parental strain 168

			B. subtilis 168 A			*B. subtilis 168 B*			*bdbCD - A*			*bdbCD- B*		
	# Cys*	# prog predict Membrane†	WT A1	WT A2	WT A3	WT B1	WT B2	WT B3	CD A1	CD A2	CD A3	CD B1	CD B2	CD B3
BdbD	2	5	8	11	13	10	11	12						
BglP	1	6	2	2	2	3	2	2						
DegS	1	1		3	3	3	3	3						
TcyP	1	4	2	2	3	3	4	2						
ProA	4	1		2	3	3	2	3						
PyrAB	8	0	8	10	12	16	14	14						
PyrAA	6	0	4	5	5	2		2						
PyrE	4	0		3	3	3	4	4						
PyrH	1	0			2		2	2						
PyrF	2	1			2	2	2	4						
YbxA	0	0	2					2						
LytA	1	4		2	2			2						
SipU	0	4			3			2						
YxaI	1	5	2					2						
GlxK	1	0			2			2						
ResD	1	1							3	3	2	4		2
CypD	8	0							2					2
MsmR	4	0							3					2

Leads were generated by comparing the detected membrane proteins of *B. subtilis* 168 and a *bdbCD* mutant. The two biological replicate experiments are indicated by A and B, each with three technical replicate injections (1-3). Low numbers of unique peptide detection are common when working with hydrophobic membrane proteins. Nevertheless, the higher the number of unique peptides identified per protein per technical replicate as indicated in the columns, the greater we considered the probability of the protein in that sample. * Number of cysteine residues within particular proteins. † Number of algorithms predicting membrane protein localization

Phenotypic assessment of BdbCD associations

One of the limits of MS analyses is that the failure to detect a particular protein does not unambiguously demonstrate its absence. Therefore, although MS is a powerful tool to identify novel leads, these leads should be confirmed at least in those cases where a suitable detection assay is available. It should be emphasized that such assays are often indirect and for many proteins no suitable assays are as yet available. This is particularly true for the membrane proteome. Nevertheless, we performed functional analyses to follow up on three potential leads for BdbCD-dependent membrane protein folding, of which at least one (i.e. ProA) was shown to be meaningful.

A number of the proteins identified here are related to cytoplasmic functions (e.g. DegS, ProA and the 5 pyrimidine-related proteins). This may imply that these proteins were possibly cytoplasmic contaminants. However, in other extensive *B. subtilis* proteomic studies these proteins have consistently been identified in the membrane fraction (184). These studies used various different extraction and MS-techniques each with their own pro's and con's when considering membrane proteomics. Hence a consistency covering not only these three studies, but the present studies performed here, suggest potential membrane-related roles for these proteins, and direct or indirect associations with BdbCD (184). Further, regarding the proteins involved in pyrimidine metabolism, most of the corresponding genes form part of a pyrimidine operon. This *pyr* operon includes the gene for the membrane -associated protein PyrP. It is noticeable that the Pyr proteins all contain a large number of cysteine residues and they thus have the capacity to form disulphide bonds as well as a Pyr protein complex at the membrane interface. Moreover, the Pyr proteins have been associated with thiol formation under oxidative stress conditions (243). The consistent membrane association and potential disulphide bond formation is thus suggestive of a membrane-associated complex. Therefore, although no functional analysis of the localization and oxidative folding of proteins involved in the pyrimidine metabolism was proven, a Bdb-Pyr relationship does deserve further in-depth investigations.

Both DegS and ResD form part of two-component regulatory systems, DegS-DegU and ResE-ResD, respectively. Contrasting results regarding these two-component systems were obtained in our studies. While DegS was observed in the membrane fraction of *the B. subtilis* 168 strain but not in the membrane fraction of the *bdbCD* mutant, the opposite was observed for ResD. The available assays for DegS and ResD are all related to their known roles in the cytoplasm. Specifically, DegS is an important regulator of motility and protease activity (244), and ResD controls expression of the ResA and

ResE proteins (245). Therefore the presence of ResA and ResE under normal growth conditions and phosphate-limited growth conditions were investigated by Western blotting, and the motility and protease activity of *bdbCD* mutants were assayed. As evidenced by the absence of the respective phenotypes (data not shown), DegS and ResD are presumably still present and active in the cytoplasm of cells lacking BdbC and BdbD.. However, as yet undefined roles of DegS or ResD at the membrane may be affected by the absence of BdbCD. Hence, the lack of detectable DegS- or ResD-related phenotypes could relate to specific roles that these proteins may be performing at the membrane interface, and this warrants further research.

The ProA protein, which was identified in membranes of the *B. subtilis* 168 strain but not in the membranes of the *bdbCD* mutant, contains four cysteine residues. ProA is involved in the synthesis of proline, an important constituent of peptides and proteins. The *bdbCD* mutant strain was therefore tested for a possible proline auxotrophy in chemically defined media. However, the mutant was able to grow normally under proline-limited conditions (data not shown). Notably, proline serves a second important role as a major osmoprotectant (246). The ability of the *bdbCD* strain to withstand osmotic shock was therefore investigated. Osmotic shock was induced by the addition of 1.1 M NaCl to exponentially growing cells and the cell viability was measured using a live-dead stain. As was to be expected for cells with significantly reduced ProA levels, the *bdbCD* strain showed a strong sensitivity to osmotic shock, and this phenotype was fully reversed when the *bdbCD* mutant was complemented through the ectopic expression of *bdbCD* from a plasmid (Figure 2).

In conclusion, our present proteomics analyses show that membrane proteomics can be applied to identify potentially TDOR-dependent membrane proteins and processes. Specifically, our studies have led to the identification of a new phenotype of *bdbCD* mutant *B. subtilis* cells, namely sensitivity to osmotic stress. This is a biologically relevant finding, because *B. subtilis* is regularly exposed to major osmotic insults in its natural habitat the soil.

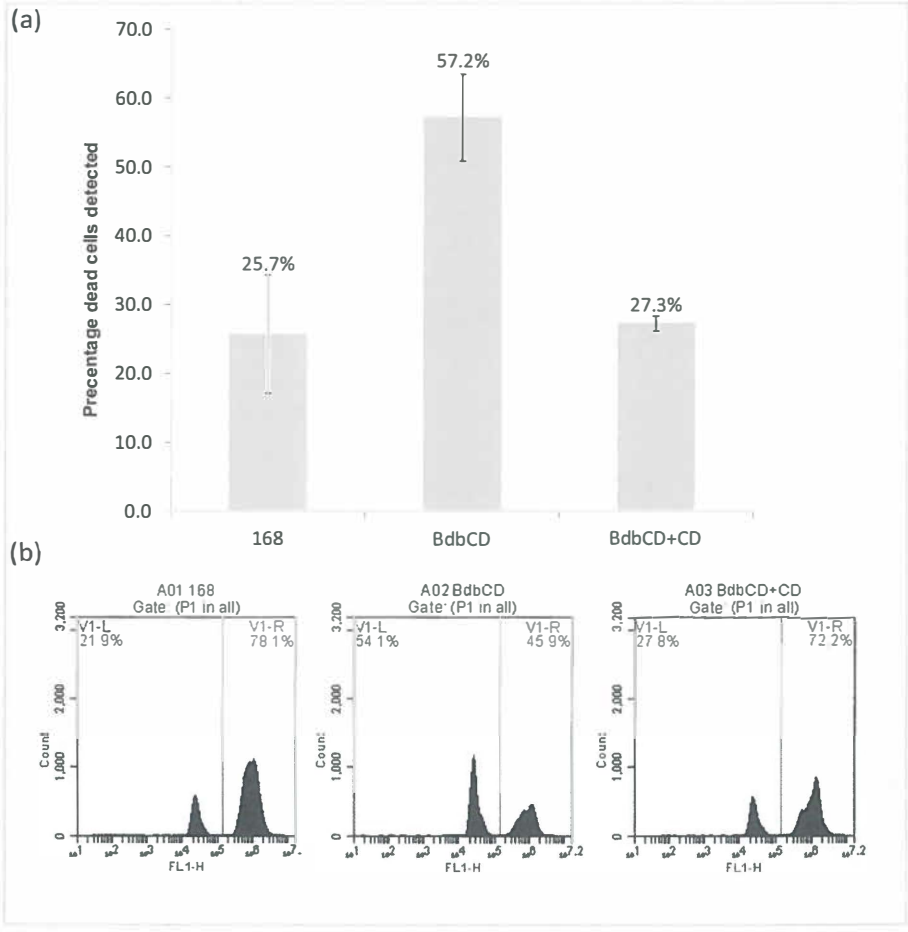

Figure 2. Increased sensitivity of *bdbCD* mutant cells to osmotic shock. The survival of cells challenged by osmotic shock with NaCl was assessed by live/dead staining and subsequent flow cytometry. (A) Percentages of dead cells detected with live/dead stain after salt shock. The *bdbCD* strain was complemented with plasmid expressing BdbC and BdbD (*BdbCD +CD*) Values represent the results of three independent experiments. The standard deviation between experiments is indicated. (B) Representative flow cytometry data indicating shifts in colour spectrum upon live/dead staining. A shift towards the left implies an increase in dead cells, where green fluorescence is measured on the x-axis and the number of cell counts on the y-axis

Notes

Bacterial strains and growth

Bacterial growth was performed at 37 °C and cultures were shaken at 250 rpm, and growth was measured by optical density readings at 600 nm. Media used in this study included Luria Bertani broth, the phosphate-limited medium LPDM (0.25% glucose, 0.21 mM KH_2PO_4 (pH 7.0), 0.025% casamino acids, 5 mM L-arginine, 1 mg Tryptophan, 50% Huletts salts [50 mM Tris pH 7, 3.03 mM $(NH_4)_2SO_4$, 6.8 mM trisodium citrate, 3.04 mM FeCl3, 1 mM $MnCl_2$, 3.5 mM $MgSO_4$, 0.01 mM $ZnCl_2$]), and the chemically defined minimal M9 medium (131) supplemented with tryptophan. When appropriate, the growth media were supplemented with 100 µg/ml spectinomycin, 2 µg/ml erythromycin, or 5 µg/ml chloramphenicol. The bacterial strains used in this study are detailed in Table 2.

Table 2. Bacterial strains used in this study

Plasmid	Properties	Reference
pHB-*bdbCD*	pHB201 vector carrying the *bdbCD* genes; Em^R; Cm	(168)
Strains	**Properties**	**Reference**
B. subtilis 168	*trp*C2	(184)
B. subtilis 168 *bdbCD*	*trp*C2; *bdbCD*::Sp^R	(168)

Membrane protein enrichment and extraction

Cultures were grown to an OD_{600} of 2. Membrane fractions were prepared as described previously (184) with minor adaptions. Protoplast disruption was performed by sonication (Soniprep 150, Beun de Ronde BV) in high salt buffer (20 mM Tris, 10 mM EDTA, 1 M NaCl). All buffers used included freshly added protease inhibitors (Complete Protease Inhibitor cocktail, Roche) except for the solubilisation buffer. The membrane protein fraction was TCA-precipitated overnight at 4°C.

LC-MS/MS and data analysis

TCA-precipitated proteins were resuspended in 8 M urea with vortexing and sonication. 100mM NH_4HCO_3 was added to the samples, which were treated with 500 mM dithiothreitol for 30 min before being incubated in the dark for 30 min with 10 µl iodoacetamide (10 mM). Trypsin digestion was performed

at 37 °C overnight with 20 µl of 250 µg/ml Trypsin, with a booster of 2-5 µl Trypsin for 1-3 h the next day before acidification with 5 % formic acid (FA).

The complex peptide mix in the samples was separated by liquid chromatography (LC) on a U-HPLC (Accela, Thermo Fisher Scientific) through a guard column (Poroshell 300 SB-C_3 2.1x12.5 mm, Agilent, Santa Clara, CA, USA) and C_{18} column-reversed phase column (Zorbax SB-C_{18} 2.1x50 mm, Agilent, Santa Clara, CA, USA) at 50 °C. Peptides were eluted at a constant flow rate of 0.4 ml/min for 275 min with a non-linear gradient 5-80 % of buffers B (Buffer A 0.1 % FA in water, buffer B 0.1% FA in acetonitrile, both UHPLC grade; Biosolve, Valkenswaard, The Netherlands).

The identification of the peptides was accomplished with an LTQ-Velos (Thermo Fisher Scientific, Bremen, Germany) coupled to an electronspray ion source. The survey scan was performed with an enhanced MS scan mass range of 300-2000. The ten most intense doubly and triply charged precursor ions were chosen for MS/MS via CID with an exclusion time of 60 s. Each sample was injected individually three times resulting in three parallel MS/MS spectra per biological replicate. The *raw files generated were visualized using Xcalibur (Thermo Fisher Scientific). This data was searched using Sorcerer-Sequest (v.27, rev. 11) (Thermo Fisher Scientific) against a *B. subtilis* 168 database including a decoy reverse database (UniprotKB, release 2011_02 – Feb, 2011). Parameters for database searches were the protease type (trypsin), variable modifications (deamination, oxidation and carbamidomethyl) and a maximum of two missed cleavage sites. Charge-dependent Xcorr factors were applied for filtering the data (2+ /3+ at 2.5/2.8) and the deltaCn value had to be at least 0.09. In addition, ambiguous peptides were excluded from the analysis. A protein was regarded as identified, if at least two unique peptides were detected resulting in a false-positive rate of below 1%.

The proteins identified by MS were compared with the predicted integral membrane proteins and potentially membrane-associated proteins of *B. subtilis*. Proteins were considered potentially membrane-associated if they were identified as membrane associated in at least 2 of 6 membrane protein prediction algorithms used (TMHMM, TMMTOP, SOSIU, PHOBIUS, SCAMPI, pSORT).

SDS-PAGE and Western blotting

Protein-separation was performed with SDS-PAGE (NuPAGE gels, Invitrogen). Gels were either stained with Simply blue™ Safe stain (Invitrogen) or semi-dry blotted onto nitrocellulose membranes (Protan, Schleicher&Schuell). Binding of polyclonal antibodies was monitored with fluores-

cent IgG secondary antibodies (IRDye 800 CW goat anti-rabbit from LiCor Biosciences) and the Odyssey Infrared Imaging System (LiCor Biosciences).

Osmostress assay

Overnight cultures in LB broth were used to inoculate fresh LB broth at a 1:200 dilution. These cultures were grown to mid-exponential phase (3 h). Samples were then diluted to an OD_{600} of 0.05 and grown to an OD_{600} of 0.4-0.5 before the addition of crystalline NaCl to a final concentration of 1.1 M. After 5 min incubation under vigorous shaking, cells were collected by centrifugation, and re-suspended in 0.85 % NaCl before a 1:1 live/dead stain was added (SYTO 9: propidium Iodide; LIVE/DEAD *Bac*Light Bacterial Viability and Counting Kit, Invitrogen). The viability of salt-stressed cells was then measured by flow cytometry (Accuri C6 Flow Cytometer).

Swarming and Swimming Assay

Swimming assays were performed on LB plates with 0.25 % agar where 2 µl of overnight culture was spotted. Swarming assays were performed on a LB plates with 0.7% agar, where before spotting 2 µl of overnight culture, 10 µl synthetic surfactin (Sigma) was spotted first. Plates were left overnight and the size of the swarming radius was viewed on the Genius:Box Documentation system using a yellow background and upper white light.

Protease activity

LB plates containing 1 % skimmed milk were spotted with 10 µl overnight culture. Zones of clearing and hence protease activity were observed after overnight growth at 37°C.

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Chapter 6

General Discussion and Concluding Remarks

General Discussion and Concluding Remarks

Protein translocation across a membrane is an essential component of many cellular functions in all kingdoms of life. However, simply moving a protein over the membrane does not imply an operational protein - functional proteins need to be correctly folded and in some instances they require co-factors. The PhD research described in this thesis was therefore focused on systems either involved in the membrane translocation of cargo proteins where folding and co-factor-attachment takes place prior to translocation, or involved in the post-translocational folding of proteins that are translocated in an unfolded state. These systems were studied in *Bacillus subtilis*. *B. subtilis* is a Gram-positive bacterium the study of which is relevant in both application-oriented and fundamental scientific settings (12-14). Notably, *B. subtilis* is one of the most well-studied and well-understood Gram-positive bacteria where global transcriptional, translational and proteomic studies have been performed (98, 130, 131, 184, 247). These studies have generated large databases allowing for unprecedented data-mining, leading to in-depth insights into Gram-positive bacterial physiology, which has allowed for the design of novel hypothesis-generating studies. Therefore the focus of this thesis was on systems relating to the folding of translocated proteins and the translocation of folded protein in *B. subtilis*.

The Sec system is the main secretion pathway in prokaryotes (1-4, 7). This pathway translocates unfolded or loosely folded proteins over the membrane, and these proteins are then tightly folded and matured in the extracytoplasmic milieu. The processes involved in this post-translocational protein maturation depend on the specific cargo protein, and they include intra-molecular folding catalysts, metal ions, isomerases, proteases and chaperones (7, 112, 120). The formation of one or more disulphide bonds between pairs of cysteine residues is essential for the functionality of certain proteins. Although these disulphide bonds can form spontaneously, specific extracytoplasmic thioldisulphide-oxidoreductase (TDOR) pathways, such as the *Bacillus* disulphide bond (Bdb) proteins in *B. subtilis*, are known to catalyse these reactions (5, 168). Bdb proteins are directly involved in the folding of proteins by catalysing disulphide bond formation (5, 171, 172). Additionally, interactions between Bdb proteins and the reductive extracytoplasmic TDORs (CcdA and ResA) have implied that the Bdb proteins are also connected to the co-factor insertion and maturation of the Sec-dependent cytochrome c_1 (QcrC) (174).

The second secretion system studied during the PhD research described in this thesis, namely the twin-arginine translocation (Tat) system, is distinctive because it is involved in the translocation of pre-folded and pre-

processed proteins. The core Tat system is broadly composed of a single membrane-spanning protein, TatC, and two TatA-like proteins most commonly referred to as TatA and TatB (36, 48). TatC and a TatA-like protein are thought to initially interact with its cargo to form a docking complex, where after numerous TatA proteins are recruited to form a pore of an appropriate size (16, 43, 44, 57, 58, 73, 74, 179). The Gram-positive bacterial Tat system was extensively reviewed in **Chapter 1**. Notably, the *B. subtilis* Tat system has been described as distinctive when compared to other Tat-systems, as it is composed of two parallel pathways each relying on a single essential TatA and TatC protein (72, 132). These two parallel pathways, TatAy-TatCy and TatAd-TatCd, each have their own substrate specificities and are transcribed under different conditions (72, 130-132). A second reason why the *B. subtilis* Tat system has been deemed unusual is that, although three TatA components were identified on the genome (TatAd, TatAy and TatAc) (185), no TatB components have been defined. Interestingly, the TatAd and TatAc proteins of *B. subtilis* have been shown to have bi-functionality in *E. coli* as they are both able to complement for the docking-complex and pore-formation roles of *E. coli* TatB and TatA respectively (23, 24).

In summary, the focus of the research described in this PhD thesis was on systems concerned with translocating folded proteins (the Tat system) and folding translocated proteins (the Bdb system). These two systems are associated with two different folding mechanisms occurring at two different sub-cellular locations. The Tat system is involved in translocating proteins that are pre-folded and receive their co-factors on the cytoplasmic side of the membrane. In contrast, the Bdb proteins are important for the disulphide bond-assisted folding of proteins on the extracytoplasmic side of the membrane.

Mass spectrometry is a powerful tool giving novel systems-level insights into Tat- and Bdb-associated proteins

Various methodologies can be used to investigate biological systems, and in this PhD research two mass spectrometric methodologies were employed to investigate both the Tat system (**Chapter 2**) and the Bdb system (**Chapter 5**). **Chapter 2** reports on quantitative proteomics analyses that were used to investigate the Tat system's interacting partners. Specific *tat* mutant strains were metabolically labelled, allowing for quantitative comparisons to be made between the different strains. Therefore, not only were a vast amount of proteins in the cytoplasm, membrane and the extracytoplasmic space identified, but this study also gave an impression of how the absence of Tat components impacted on the global proteome. This quantita-

tive data was approached in both hypothesis-driving and -driven manners. In the hypothesis-driving approach, the data set was studied purely objectively, and due to the ability of the technique to quantify proteins, slight variations between the amounts of proteins implicated a number of novel Tat-affected proteins. Of these numerous proteins, the included proteins of interest were involved in iron-sulphur cluster assembly (SufS) and cytoplasmic protein folding (DnaJ). However, in the absence of defined biochemical or physiological assays most of these potential Tat-associated interactions could not be independently confirmed. Nevertheless, a number of proteins linked to biofilm formation were reduced in strains without the TatAy-TatCy pathway. This led to the identification of a novel Tat-associated delayed biofilm phenotype of *B. subtilis*. This phenotype could be due to a still unidentified Tat-dependent substrate or indirect ramifications caused by a Tat-deficiency. In the hypothesis-driven approach, proteins that had previously been linked to the Tat system were exclusively investigated. Importantly, using this hypothesis-driven methodology, the Tat-dependent substrate QcrA was identified. The Rieske iron-sulphur protein QcrA had been missed in the hypothesis-driving approach due to necessary statistical constraints. By combining both the hypothesis-driven and -driving results, it is tempting to argue that the decreased amount of SufS protein in *tatAy-tatCy* mutant strains as observed in the hypothesis-driving approach may be linked to the absence of the iron-sulphur containing QcrA protein from these strains. However, this is still to be proven. Further, QcrA turned out to be an excellent example of how approaching the MS-data set from two angles enriched our deductions, and the subsequent detailed studies on the Tat-dependent membrane translocation of QcrA are documented in **Chapter 4** of this thesis.

The second mass spectrometric study focused on the Bdb system. **Chapter 5** presents the analyses in which the presence or absence of proteins was examined in strains where the chief extracellular oxidative redox pair was removed, namely BdbC-BdbD. The focus of this study was specifically the membrane proteome, as previous proteomic studies had not identified new Bdb-dependent substrates in the extracytoplasmic fraction (Haike Antelmann and Jan Maarten van Dijl, unpublished observations). When comparing the membrane proteome of the wild-type *B. subtilis* strain 168 and the *bdbC-bdbD* mutant strain a number of proteins appeared or disappeared. These differences suggested a number of potential BdbC-BdbD interactions, but did not directly implicate a substrate. Nonetheless, proteins involved in pyrimidine synthesis were absent from the *bdbC-bdbD* mutant and an osmotic shock phenotype consistent with the absence of the glutamate-5-semialdehyde dehydrogenase ProA involved in proline biosynthesis was unveiled.

Comparisons between the translocation of Tat-dependent cargo proteins allow for novel hypotheses into the roles of *B. subtilis* Tat components in translocation.

The basic components of the *B. subtilis* Tat system were confirmed when starting this thesis, as the TatAd-TatCd and TatAy-TatCy pathways were shown to translocate PhoD and EfeB (YwbN) respectively (25, 72). However, no clear chaperone proteins or quality control mechanisms had been observed in *B. subtilis* and, although a larger number of substrates were predicted, only two Tat-dependent cargo proteins were confirmed. Further, a role for the third TatA protein, TatAc, had remained enigmatic. Over the subsequent years new Tat-dependent cargo proteins have been identified and studied (i.e. YkuE (129) and QcrA; **Chapters 2** and **4**), and a number of Tat-associated protein-protein interactions have been shown (HemAH, CsbC and WprA) (98). The *B. subtilis* Tat-system was further proven to not only translocate secreted proteins, but it was also shown to translocate proteins destined for the membrane (i.e. QcrA) and cell wall (i.e. YkuE; (20, 125, 129) and **Chapter 2**). Further, TatAc's ability to translocate Tat-dependent cargo proteins when expressed with TatCy or TatCd was shown in *E. coli* (87). This increase in the knowledge of the *B. subtilis* Tat system allowed for the development of assays, new hypotheses, and the recognition that the Tat system is involved in membrane protein insertion and cell wall association. Ultimately, this has led to a better understanding of the *B. subtilis* Tat system.

Previous studies had generated invaluable strains where specific amino acid residues in the TatAy and TatCy proteins were mutated (68, 83). These strains were investigated in detail in this PhD research. The assay described in **Chapter 3** took advantage of an EfeB-associated lysis phenotype. In this assay, cell lysis was used as a read out for the level to which translocation of active EfeB was impaired by particular *tat* mutations. Importantly, the observed lysis phenotypes were distinct and could be graded, allowing for unusually detailed insights into the importance of specific amino acid residues in TatAy or TatCy. Mild phenotypes were suggestive of a weakened translocation of active EfeB, while severe and very severe lysis phenotypes suggested more serious defects. Interesting observations made when comparing the phenotypes generated by strains that no longer produced TatAc showed that some phenotypes worsened. By re-introducing TatAc this worsening phenotype was often reversed. Previously no phenotypes have been observed in strains where TatAc was deleted, nor could TatAc on its own functionally replace TatAy or TatAd (14, 25, 72). Therefore, the results in this study imply that TatAc assists TatAy in the translocation of EfeB and, if necessary, is able to partially replace TatAy as long as mutated TatAy is still present.

Phylogenetic analyses where the Tat systems from all Kingdoms were compared have shown that Tat systems are most often composed of two TatA components and one TatC component (36, 48). The two TatA-like proteins have often evolved into specialised functions, namely pore formation (TatA in *E. coli*) and docking complex formation (TatB in *E. coli*) (43, 44, 57, 58, 73, 74). Hence, one could hypothesise that *B. subtilis* TatAc behaves much like the TatA protein in *E. coli* and functions predominantly as a pore forming TatA-like protein, while *B. subtilis* TatAy behaves much like the TatB protein in *E. coli* in that it is involved in docking-complex activities. However, *B. subtilis* TatAy (and also TatAd) are both able to perform pore and docking-complex functions (23, 24, 72). Therefore, although TatAc may look like a superfluous assistant pore-forming protein in *B. subtilis,* it does seem to participate actively in protein translocation. Altogether, these findings indicate that the three *B. subtilis* TatA proteins represent evolutionary intermediates of the TatA-TatB proteins observed in other bacteria (figure 1).

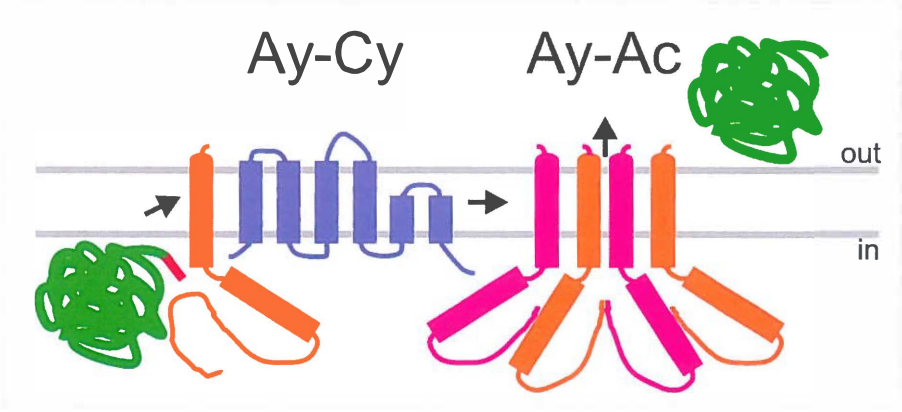

Figure 1. The *B. subtilis* TatAy-TatCy-TatAc translocation system. TatAy combined with TatCy to from a docking complex with the cargo protein, where after more TatAy and TatAc proteins are recruited to form the translocation pore. TatCy is indicated in purple, TatAy in orange, TatAc in pink, a cargo protein in green and the cargo protein's RR-signal peptide in red.

Although site-specifically mutated TatAy and TatCy proteins were initially used to explore the role of TatAc in the *B. subtilis* Tat system, the strains producing these mutant proteins were also used for studies described in **Chapters 3** and **4** to compare the translocation profiles of different cargo proteins, namely EfeB (**Chapter 3**) and QcrA (**Chapter 4**). It was

shown that specific residues in TatCy and the hinge region of TatAy are important for translocation of both EfeB and QcrA. Interestingly, certain other residues in the amphipathic helix region of TatAy showed cargo protein-specific variations. In *E. coli* the TatC-TatB docking complex has been implicated in cargo protein proofreading and recognition (11, 16, 40-43, 225). There is no discernable TatB in *B. subtilis* and the *B. subtilis* TatA proteins have been implicated in bifunctional TatA-TatB activity (23, 24). It is therefore possible that the cargo-specific effects of mutations in the amphipathic helix region of TatAy are related to proofreading and recognition functions of the TatAy-TatCy docking complex.

Combining the hypothesis presented in **Chapter 3** with regard to TatAc's potential pore-forming role and the TatAy cargo-specific interactions presented in **Chapters 3** and **4**, suggests that TatAy has a dual pore/docking-complex role, while TatAc assists TatAy in the pore-complex formation. This hypothesis is based on our current understanding of the Tat-pathway. However, the roles of individual Tat-components in translocation are still somewhat contested. In particular, it is not known exactly when and how cargo proteins specifically interact with Tat pathway components, how the pore is formed, or how large the pore precisely is. Moreover, the proposed mechanisms involved in the translocation and release of the cargo proteins have not been unequivocally proven. Therefore, further studies expanding inter-Tat-interactions are warranted. Yeast-two-hybrid (Y2H) studies have previously shown that the three different *B. subtilis* TatA proteins interact with themselves and each other (98). Additional Y2H studies investigating the interactions between TatAc, TatAd and the site-specifically mutated TatAy proteins described in **Chapters 3** and **4** could potentially lead to a better understanding of essential inter-Tat-relationships.

The newly identified Tat-dependent cargo protein QcrA provides unprecedented insights into the quality control requirements of the *B. subtilis* Tat system

In the studies described in **Chapter 4**, the importance of folding and co-factor insertion for successful Tat-dependent translocation in the Rieske iron-sulphur protein QcrA was investigated. Rieske iron-sulphur proteins, such as QcrA, contain both a co-factor and have a disulphide bond essential for folding. Therefore, by introducing mutations in QcrA that disrupt either the co-factor insertion or disulphide bond formation, the consequences of these protein-folding requirements could be dissected. It was shown that both co-factor insertion and correct disulphide-assisted folding were vital for full translocation, but importantly, a hierarchy in quality control between these two requirements was observed. A mutant protein unable to form the

disulphide bond was immediately degraded, most likely in the cytoplasm. In contrast, mutant proteins defective in co-factor binding were detectable in the cytoplasm and membrane.

The results presented in **Chapter 4** are the first to describe a cytoplasmic quality control mechanism for *B. subtilis*. However, the exact mechanisms responsible for this quality control are still unknown and sought after. There are various experiments that could be performed to further investigate the degradation of the QcrA mutant defective in disulphide bonding. Co-factor mutated QcrA proteins could be further investigated as to where their membrane translocation is impaired, i.e. on the cytoplasmic side of the membrane, within the membrane or on the extracytoplasmic side of the membrane. This could be achieved by treating protoplasts with trypsin and observing the sensitivity of QcrA co-factor mutants to trypsin. Further, investigations regarding cytoplasmic proteases would also be of interest. ClpP is a cytoplasmic protease central to the cytoplasmic proteolytic system (248). Hence expressing the QcrA mutant proteins in a genetic background without ClpP might give interesting insights regarding quality control mechanisms of this Tat-dependent substrate.

The studies on the QcrA mutants described in **Chapter 4** make it tempting to speculate that the Tat system is directly involved in QcrA quality control mechanisms. However, a direct link between the Tat system and the degradation of misfolded QcrA was not shown. In order to address this, an experiment where in a double *tat-qcrA* mutant background the QcrA-associated mutants are introduced would provide direct insights into the possible role of the Tat-system in QcrA quality control.

Concluding remarks

Our understanding of systems involved in protein folding and the secretion of folded proteins in *B. subtilis* has been enriched by the work presented in this thesis. The membrane proteome of a BdbC-BdbD-deficient strain was investigated, leading not only to a compendium of potentially associated proteins, but also establishing a link between the BdbC and BdbD proteins and the osmotic shock protective system of *B. subtilis*. Until the research presented in this study was done, the only known native BdbC- and BdbD-associated proteins of *B. subtilis* were those involved in the binding and uptake of DNA during genetic competence. However, it was now clearly illustrated that the absence of the BdbC and BdbD proteins causes distinct changes in the membrane proteome. This implies interactions and effects associated with the Bdb system. Further investigations into the membrane proteins affected by

BdbD and BdbC could lead to defining novel protein-protein interactions and thereby expanding our understanding of oxidative protein folding and broader bacterial physiology.

The Tat system was the second, major, focus of this thesis. The work presented here addressed a number of questions regarding *B. subtilis* Tat-dependent cargo proteins, the quality control mechanisms and the role of TatAc in translocation. The investigations in this study led to the generation of large mineable dataset with Tat-associated quantitative proteomic changes, the identification of a new cargo protein, the confirmation of quality control requirements associated with this cargo protein, and importantly, deciphering of the role of TatAc in *B. subtilis*. However, as is the nature of science, by addressing these questions inevitably more were generated. The Tat pathway remains an enigmatic secretion system, and although we know more, the exact mechanisms of translocation and quality control are not yet known. Importantly, the Tat system has both applications in industry, and is central to the virulence of certain pathogenic bacteria, including the causative agent of tuberculosis. Hence, further research into this fascinating translocation system could ultimately lead to completely novel applications in biomanufacturing or the treatment of major bacterial infectious diseases.

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Appendices

Nederlandse Samenvatting voor de leek

Inleiding:

Alle levende organismen zijn opgebouwd uit cellen. Een cel is in essentie een waterig compartiment - het cytoplasma - met daarin alle levensnoodzakelijke bestanddelen. Het cytoplasma wordt omsloten door een dunne membraan die uit een fosfolipide dubbellaag bestaat. Deze laag houdt het cytoplasma gescheiden van het extracellulaire milieu. Door processen van osmose en diffusie kunnen kleine hydrofobe moleculen spontaan de membraan passeren, maar voor grotere hydrofiele moleculen vormt de fosfolipide dubbellaag een ondoordringbare barrière. Het transport van dergelijke moleculen over het membraan is echter van fundamenteel belang voor het leven van een cel. Invoer van moleculen van buiten naar binnen is essentieel voor de stofwisseling en het genereren van energie. Ook verschaft dit transport een cel vitale informatie over de veranderende omstandigheden, waarin hij zich bevindt. Om verschillende redenen is de export van moleculen van binnen naar buiten minstens even belangrijk als de import. In de eerste plaats is export nodig voor de verwijdering van afvalstoffen. Daarnaast zijn exportprocessen, met name de eiwitexport, ook onmisbaar voor de assemblage van opnamesystemen voor voedingsstoffen, het waarnemen van milieuveranderingen, cel-cel interacties en, in het geval van ziekteverwekkers, de secretie van virulentiefactoren of toxines.

Eiwitten zijn de belangrijkste actieve moleculen in een cel, waarbij afzonderlijke eiwitten hun eigen specifieke functies hebben die gezamenlijk het overleven van de cel als geheel verzekeren. In sommige gevallen moeten eiwitten in de membraan of zelfs buiten de cel geplaatst worden om hun specifieke functie naar behoren uit te voeren. Deze extracytoplasmische eiwitten moeten daarom actief door de membraan getransporteerd worden. De cel heeft hiervoor een set specifieke eiwitten – eiwit-transporteurs - die de translocatie van andere eiwitten mogelijk maken.

Een juiste lokalisatie is niet de enige voorwaarde voor de activiteit van een eiwit. Eiwitten zijn gemaakt van ketens van aminozuren. Om zowel stabiel als actief te zijn moeten deze ketens op de juiste manier gevouwen

worden. In sommige gevallen is voor eiwitactiviteit en stabiliteit ook nog de binding van extra co-factoren nodig.

Binnen de cel zijn een aantal systemen betrokken bij eiwitexport. De belangrijkste route voor eiwitexport wordt gevormd door het algemene secretie (Sec) systeem. Eiwitten die de Sec-route volgen worden uitgevoerd in een niet-gevouwen toestand en na de membraantranslocatie moeten deze eiwitten daarom alsnog gevouwen worden. Verschillende systemen zijn geëvolueerd om dit vouwingsproces te begeleiden en de zogenaamde Thioldisulfide-oxidoreductases (TDORs) hebben hierbij een belangrijke taak. De TDORs zorgen namelijk voor de vorming van disulfidebruggen die de gevouwen vorm van een eiwit kunnen fixeren. Er zijn echter ook eiwitten die een alternatief exporttraject volgen en daarbij in een volledig gevouwen toestand de membraan passeren. Een dergelijke route die in bacteriën en in de thylakoiden van chloroplasten in groene planten voorkomt is de zogenaamde Twin-arginine translocatie (Tat) route. De translocatie van zo'n volledig gevouwen eiwit is intrigerend, want de poriën die nodig zijn om een gevouwen eiwit over het membraan te transporteren moeten relatief groot zijn. Dit vergroot het risico voor verlies van belangrijke componenten die in de cel moeten blijven en het is daarom noodzakelijk om het eiwittransportproces via de Tat-route zorgvuldig te controleren.

Bacillus subtilis is een Gram-positieve bacterie met het vermogen om grote hoeveelheden eiwit te secreteren. Veel gesecreteerde enzymen van *Bacillus*-soorten, zoals *B. subtilis*, hebben een hoge commerciële waarde. Verder heeft *B. subtilis* een lange geschiedenis van veilig gebruik voor biotechnologische doeleinden. Om deze redenen is *B. subtilis* uitgegroeid tot een 'werkpaard' voor de bio-industriële productie van eiwitten. Tegelijkertijd is *B. subtilis* ook een belangrijk modelorganisme geworden voor fundamenteel onderzoek, met name naar de mechanismen die Gram-positieve bacteriën gebruiken voor de secretie en vouwing van gesecreteerde eiwitten. De focus van het onderzoek beschreven in dit proefschrift was dan ook de analyse van de *B. subtilis* systemen voor export van gevouwen eiwitten (de Tat-route) en de vouwing van uitgescheiden eiwitten (het TDOR-systeem). Omdat het TDOR-systeem van *B. subtilis* betrokken is bij de vorming van disulfidebruggen worden de eiwitten die dit systeem vormen ook wel de Bdb (*Bacillus* disulfide-binding) eiwitten genoemd.

Resultaten van het onderzoek

Twee aparte studies beschreven in dit proefschrift zijn uitgevoerd met behulp van vergelijkbare technieken gebaseerd op de zogenaamde massaspectrometrie. Massaspectrometrie is een krachtige techniek voor het identificeren van eiwitten en deze techniek werd daarom gebruikt voor de dissectie van het Tat-systeem (**hoofdstuk 2**) en het TDOR-systeem van *B. subtilis* (**hoofdstuk 5**). In beide studies werd de massaspectrometrische analyse uitgevoerd op *B. subtilis* stammen met specifieke mutaties, waardoor ze of defecten in het Tat-systeem of in het TDOR-systeem hadden. De eiwitprofielen van de Tat- of TDOR-mutanten werden vergeleken met het eiwitprofiel van de oorspronkelijke niet-gemuteerde stam. Op die manier werden verschillende afhankelijkheidsrelaties waargenomen. Deze nieuwe relaties worden in detail beschreven in de **hoofdstukken 2** en **5** van dit proefschrift.

De individuele componenten van het Tat-systeem en hun specifieke rollen in eiwittranslocatie waren de focus van het onderzoek beschreven in **Hoofdstuk 3**. Het Tat-systeem van *B. subtilis* bestaat uit verschillende eiwitten die bekend staan onder de verzamelnamen TatA en TatC. Van één van de drie *B. subtilis* TatA-eiwitten, TatAc, was de rol bij aanvang van dit promotieonderzoek nog totaal onduidelijk. In **hoofdstuk 3** wordt aangetoond, dat TatAc het zogenaamde TatAy-eiwit ondersteunt bij de export van het zogenaamde EfeB-eiwit. De waargenomen functionele interactie tussen TatAc en TatAy is nieuw, maar doet in sterke mate denken aan de eiwitinteracties die waargenomen worden in de Gram-negatieve bacterie *Escherichia coli* die nog een extra Tat-eiwit bezit, dat bekend staat onder de naam TatB. Hoewel TatB in *B. subtilis* en vele andere Gram-positieve bacteriën niet voorkomt lijkt het er op grond van de huidige resultaten op, dat TatAc zich gedraagt als een evolutionaire tussenvorm van TatA en TatB.

De massaspectrometrische en biochemische analyses beschreven in **hoofdstuk 2** hadden laten zien, dat het zogenaamde QcrA-eiwit via de Tat-route in de cytoplasmamembraan van *B. subtilis* geïnsereerd wordt. Dit proces werd verder onderzocht in de analyses die beschreven zijn in **hoofdstuk 4**. De resultaten laten zien dat co-factor-binding een voorwaarde is voor efficiënte membraaninsertie van QcrA. De vorming van een disulfidebrug in QcrA is zelfs nog belangrijker want een gemuteerd QcrA-eiwit, dat de disulfidebrug niet kan vormen, werd zeer snel afgebroken. Dit wijst op een hiërar-

chie in de kwaliteitscontrole van het te exporteren QcrA-eiwit die nog niet eerder was beschreven.

Conclusie

Samenvattend kan geconcludeerd worden, dat het onderzoek beschreven in dit proefschrift onze kennis over de systemen voor eiwitvouwing en export van gevouwen eiwitten verrijkt hebben. Voor het eerst werd bijvoorbeeld een verband waargenomen tussen de TDOR-gemedieerde eiwitvouwing en resistentie tegen osmotische stress. Op grond van de massaspectrometrische analyses is deze connectie mogelijk terug te voeren op een beperkt aantal eiwitten van *B. subtilis*, waaronder het ProA-eiwit. Daarnaast bleek het Tat-systeem van invloed op de vorming van zogenaamde biofilms door *B. subtilis*. Ook dit inzicht is voortgekomen uit de massaspectrometrische analyses, waarbij bleek dat meerdere eiwitten die een rol hebben bij biofilm-vorming beïnvloed worden door Tat-mutaties. Heel specifiek werd het QcrA-eiwit geïdentificeerd als een eiwit, dat Tat-afhankelijk geëxporteerd wordt uit de *B. subtilis* cel en dit inzicht bood vervolgens de mogelijkheid om het proces van QcrA-export in meer detail te bestuderen. Tenslotte werd de rol van het TatAc eiwit bij de Tat-afhankelijke eiwitexport door *B. subtilis* opgehelderd. Hoewel een aantal belangrijke vragen over het Tat-systeem van *B. subtilis* in het onderhavige onderzoek zijn beantwoord, blijft er toch nog veel over om in meer detail te onderzoeken. In dit opzicht is het van belang, dat het Tat-systeem enerzijds toepassingen kan vinden bij de industriële productie van eiwitten, maar anderzijds ook een centrale rol speelt in de virulentie van bepaalde pathogene bacteriën, waaronder de veroorzaker van tuberculose. Verder onderzoek naar het moleculaire mechanisme van dit fascinerende systeem voor eiwittransport zou derhalve zowel kunnen leiden tot geheel nieuwe biotechnologische toepassingen als ook tot de bestrijding van belangrijke infectieziekten.

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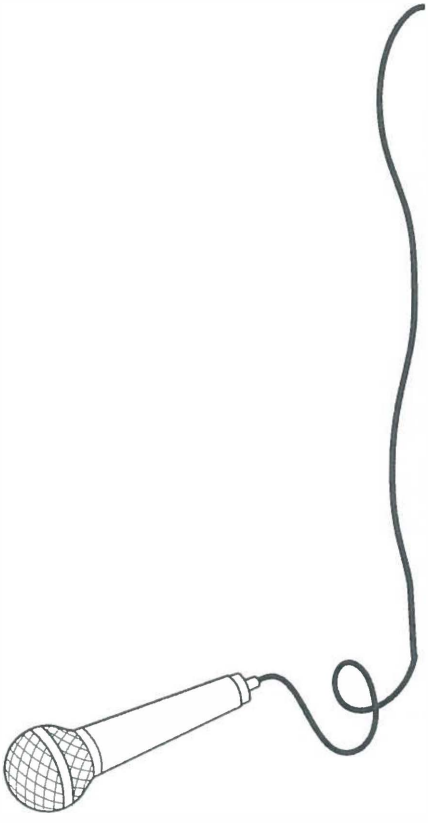

And that's how Big Girls do it.

List of Publications

Goosens VJ, Mars RA, Akeroyd M, Vente A, Dreisbach A, Denham EL, Kouwen TR, van Rij T, Olsthoorn M, van Dijl JM. **Is Proteomics a Reliable Tool to Probe the Oxidative Folding of Bacterial Membrane Proteins?** *Antioxid Redox Signal.* 2013 Apr 1;18(10):1159-64.

Goosens VJ, Monteferrante CG, van Dijl JM. **The Tat system of Gram-positive bacteria.** *Biochim Biophys Acta, In press.*

Goosens VJ, Otto A, Glasner C, Monteferrante CG, van der Ploeg R, Hecker M, Becher D, van Dijl JM. **Novel twin-arginine translocation pathway-dependent phenotypes of Bacillus subtilis unveiled by quantitative proteomics.** *J Proteome Res.* 2013 Feb 1;12(2):796-807.

Goosens VJ, van Dijl JM. **Co-factor insertion, disulphide bonding and Tat translocase requirements for membrane translocation of the Bacillus subtilis Rieske protein QcrA.** Submitted to *J Biol Chem*

Krishnappa L, Dreisbach A, Otto A, Goosens VJ, Cranenburgh RM, Harwood CR, Becher D, van Dijl JM. **Extracytoplasmic proteases determining the cleavage and release of secreted proteins, lipoproteins and membrane proteins in Bacillus subtilis.** *J Proteome Res.* 2013 Aug 13

Moola N, Goosens VJ, Mizrahi V, Kana BD, Gordhan BG. **The Nei and Nth DNA glycosylases are required for mutagenesis in *Mycobacterium smegmatis*.** In press - submitted August 2013 to *DNA repair*

van der Ploeg R, Barnett JP, Vasisht N, Goosens VJ, Pöther DC, Robinson C, van Dijl JM. **Salt sensitivity of minimal twin arginine translocases.** *J Biol Chem.* 2011 Dec 23;286 (51): 4375